THE CYTOTOXIC EFFECTS OF ASBESTOS FIBRES ON P388D$_1$ CELLS AND MACROPHAGES IN VITRO.

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DECLARATION.

The results presented in this thesis have been submitted in part fulfilment of the requirements for the degree of PhD; they have not been submitted for any other degree, and, except where due acknowledgement has been given, both the results and the manuscript are of the author's own composition and work.

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ABBREVIATIONS.

A	amp
Å	angstrom
AMP	adenosine monophosphate
ARC	Asbestosis Research Council
BP	benzo(a)pyrene
°C	degrees Centigrade
Ca^{++}	calcium ion
Cl^{-}	chloride ion
cm	centimetre
CMC	carboxymethyl cellulose
CO_2	carbon dioxide
Con A	concanavalin A
C parvum	Cornynebacterium parvum
cwt	hundred weight
DC	direct current
diam	diameter
DNA	deoxyribonucleic acid
E	elutriated
EGTA	ethylene glycol-bis-(β-aminoethylether) N,N'-tetraacetic acid
EM	electron microscope
F	factory
F	Farad
g	unit of acceleration of gravity
GMP	guanosine monophosphate
H	heated
HCl	hydrochloric acid
H_2O	water
hr	hour
IOM	Institute of Occupational Medicine
IR	input resistance
K^{+}	potassium ion
KCl	potassium chloride
l	litre
LDH	lactate dehydrogenase

LF	long fibre
log	logarithm
M	molar
ME	microelectrode
mg	milligram
Mg^{++}	magnesium ion
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
Mn^{++}	manganese ion
$MnCl_2$	manganese chloride
MΩ	megohms
MOPS	morpholinopropanesulfonic acid
MP	membrane potential
MRC	Medical Research Council
mV	millivolt
MW	molecular weight
N	normal
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NHMRC	National Health Medical Research Council
nm	nanometre
no	number
OD	optical density
$P388D_1$	mouse lymphoma cell line
PAH	polycyclic aromatic hydrocarbon
PBS	phosphate buffered saline
PEC	peritoneal exudate cell
PH	parent of heated
ppm	part per million
PRU	Pneumoconiosis Research Unit
PVPNO	polyvinyl pyridine-N-oxide
SD	standard deviation
sec	second
SEM	scanning electron microscope

SF	short fibre
TEM	transmission electron microscope
TV	television
UICC	Union Internationale Contre le Cancer
V	volt
VBS	veronal buffered saline
WDC	wet dispersed chrysotile

Symbols:

µg	microgram
µl	microlitre
µM	micromolar
µm	micron
>	greater than
<	less than
%	percentage
λ	wavelength
ε	extinction coefficient

ABSTRACT.

Asbestos is a term used to encompass a number of fibrous minerals that are obtained by mining and are used extensively in industry to produce a variety of commercially available products. The exposure of man, by inhalation, to the airborne form of these fibres results in the development of such disease states as asbestosis, bronchial carcinoma or mesothelioma. The alveolar macrophage is primarily involved in defending the lung against the invasion of inhaled pathogens, and has the ability to phagocytose and clear particulate material, destroy biological material and communicate with the immunological system. The aim of this study was to investigate the effects of various types of asbestos on macrophages in vitro, in particular with regard to the involvement of the fibre dimensions in determining the cytotoxic potential of each sample. In order to examine the characteristics of each asbestos type, a technique using scanning electron microscopy was established which allowed the ready assessment of the dimensions and fibre number content of each sample. The $P388D_1$ macrophage-like cell line was used for the routine examination of asbestos/cell interactions. The ability of each asbestos sample to modify cellular viability, lactate production and rate of release of lactate dehydrogenase and glucosaminidase was established 24 and 48 hrs following exposure of $P388D_1$ cells to dust concentrations of 10 or 50 μg/ml. In general, a close association was seen between the reduction in cell viability and loss of intracellular enzyme following the treatment of cells with a dust sample. The serpentines proved more cytotoxic than the amphiboles, and those chrysotile samples prepared by the industrial wet-dispersal process displayed the greatest degree of cytotoxicity towards $P388D_1$ cells. In general a close association between the cytotoxic potential of a fibrous sample and the number of fibres greater than 8 μm in length was observed. The ability of various asbestos samples to modify the $P388D_1$ cell membrane electrical activity was established by measuring the membrane potential and membrane resistance. In general, the observed trend was similar to that seen following the cytotoxicity analysis, as the more cytotoxic dust samples caused a rapid decline in both membrane potential and resistance. The amosite samples,

with the exception of the short fibre amosite material, showed the ability to elicit a cell membrane hyperpolarisation which was due to the activation of a calcium sensitive potassium conductance channel. Finally, the *in vitro* cytotoxicity of the three UICC asbestos samples towards mouse peritoneal macrophages in various activational states was examined. UICC amosite and crocidolite proved to be inactive towards all types of macrophage. The degree of susceptibility to the cytotoxic action of chrysotile varied; the more activated populations proved more susceptible than the resident population and in particular, those populations activated *in vivo* following intraperitoneal injection of UICC asbestos showed the greatest degree of susceptibility to chrysotile.

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1.1 An Historical Background to the Use of Asbestos

Asbestos has been used by man since prehistoric times, and as early as 2,500 B.C. the fibre was employed in Finland to strengthen pottery and block crevices in log huts (Selikoff and Lee, 1978). In records written by Plutarch and Pausanius (46 - 120 A.D.) and Pliny (23 - 79 A.D.) it was noted that asbestos was used for perpetual lamp wicks and also woven to form the "funeral dress of kings" (Harington, 1976), thereby illustrating that the non-combustible nature of the mineral had been discovered. Asbestos usage continued during the Middle Ages for the production of such articles as lamp wicks and paper in Norway, handkerchiefs in China, and leggings, ropes, belts and bags in the Pyrenees (Selikoff and Lee, 1978).

The enormous commercial potential for asbestos was eventually realised in the late nineteenth century, when a number of deposits of the mineral were discovered in Canada and S. Africa. At the same time, the need for an insulating agent for steam engine boilers emerged (Gilson, 1973), and it was recognised that asbestos would satisfy this need. The production of asbestos started relatively slowly (for example 50 tons per annum from Quebec in 1878), but escalated as the number of uses for the fibre increased (i.e. 911,226 tons from Quebec in 1953) (Bowles, 1955). Eventually by 1970 the world's asbestos output exceeded 4 million tons per year (Clifton, 1972).

Unfortunately, the increase in commercial output and usage of asbestos also involved an increase in both the occupational and the domestic exposure of the population to asbestos fibres. In 1907 a link between lung disease and asbestos exposure was reported (Murray, 1907), and by 1930 it was confirmed that asbestos posed a considerable threat to the exposed individual (Merewether and Price, 1930). The number of reports citing associations between asbestos exposure and increased incidence of lung carcinoma, asbestosis, mesothelioma and other cancers, escalated (see reviews by Selikoff and Lee, 1978; Becklake, 1976). These findings resulted in a considerable interest of both the public and commercial sectors in the pathogenicity of asbestos towards man. Extensive research evolved towards an examination of the nature of asbestos, the generation of the

asbestos hazard, the degree of pathogenicity of asbestos, and the mechanism by which asbestos initiates disease. These topics will be reviewed in the following sections.

1.2 Asbestos Types and Their Structure

The term "asbestos" is generally used to encompass a number of naturally occurring, rock-forming minerals known as fibrous silicates. Six types of asbestiform mineral are of commercial importance and these belong to the serpentine and amphibole groups. They are grouped and classified according to their chemical composition (Table 1.1) and comprise, in decreasing order of commercial importance: chrysotile, crocidolite, amosite, anthophyllite, tremolite and actinolite (Miller, 1978). Chrysotile is the only type belonging to the serpentine group, and the crystalline structure of both chrysotile and the amphiboles is complex.

1.2.1 The Structure of Chrysotile

The crystalline structure of chrysotile comprises a layer of magnesium-hydroxide octahedra (a brucite layer) bonded to a layer of silicon-dioxide tetrahedra (Warren and Herring, 1941). The dimensions of the two sheets do not match and the structure becomes strained. The degree of strain is relieved by curving the whole of the double sheet, so that the brucite layer is on the outer surface (Warren and Herring, 1941). The individual fibrils consist of a spiral tube (Yada, 1967), and each fibril binds together to form the curly chrysotile fibres (Deer et al, 1962, 1963). In general the composition of chrysotile does not vary from $Mg_3(Si_2O_5)(OH)_4$, although impurities such as iron, or aluminium, and occasionally calcium, chromium, nickel, manganese, sodium and potassium may substitute in one or other of the layers (Reimschussel, 1975).

1.2.2 The Structure of the Amphiboles

The amphibole ultrastructure comprises double chains of linked silicon oxygen tetrahedra which lie parallel to the vertical crystallographic axis and are bound laterally by metallic ions (Speil and Leineweber, 1969). This structure does not roll into a tube, as seen with chrysotile. The Si-O bonds along the chains are stronger than the metallic ion bonds between them, and thus the

amphiboles tend to fracture lengthwise and form straight fibres (Speil and Leineweber, 1969).

In addition to those ions shown in Table 1.1 to be present in the crystalline lattice of the various asbestos types, a number of contaminating, trace elements may also be found (Morgan and Cralley, 1973) (Table 1.2). The presence of these ions may be due to the replacement of certain cations or contamination with free impurities during the milling process. In addition a number of naturally occurring oils and waxes (Harington, 1962), and various contaminating oils and hydrocarbons adsorbed during the manufacturing process, have also been identified (Harington, 1962; Harington et al, 1975).

From the preceding sections it is evident that the various asbestos types may differ considerably from each other, depending upon their respective ionic contents and chemical structures. Such heterogeneity results in a multiplicity of physical and chemical properties which render each of the asbestos types unique in their respective commercial usefulness. It must also be borne in mind that those properties of asbestos which have rendered them of commercial importance may also be of considerable biological significance, as the different asbestos types may elicit a different response in a number of biological interactions.

1.3 Some Commercially Important Properties of Asbestos

The major properties that have ensured the continuation of the use of asbestos are their flexibility, surface properties, chemical reactivity, strength and thermal reactivity.

1.3.1 Flexibility and Fibre Dimensions

The ability to spin and weave asbestos is of importance in the textile trade, and this is dependent upon a combination of the flexibility and length of the fibre (Selikoff and Lee, 1978). In general, the softer chrysotiles, crocidolite and some amphiboles (depending upon their origin) are flexible, whereas the remaining amphiboles are relatively brittle (Harington et al, 1975). The length of the asbestos fibres may vary according to the type, and these are in order of decreasing length: amosite (15 - 30 cm) > crocidolite (17 cm) > chrysotile (5 cm) > anthophyllite which is relatively short and weak

Table 1.1 Classification of Asbestos Minerals

GROUP	VARIETY	FORMULA
amphibole	anthophyllite	$Mg_7Si_8O_{22}(OH)_2$
	amosite (brown)	$(Fe^{2+}Mg)_7Si_8O_{22}(OH)_2$
	crocidolite (blue)	$Na_2Fe^{3+}(Fe^{2+}Mg)_3Si_8O_{22}(OH)_2$
	tremolite	$Ca_2Mg_5Si_8O_{22}(OH)_2$
	actinolite	$Ca_2(Mg\ Fe^{2+})_5Si_8O_{22}(OH)_2$
serpentine	chrysotile (white)	$Mg_3(Si_2O_5)(OH)_4$

After Michaels and Chissick (1979).

(Selikoff and Lee, 1978). Chrysotile, however, has the finest diameter fibrils of 0.02 μm. (Berger, 1963) compared to 0.06 μm for crocidolite, 0.15 μm for amosite and 0.25 μm for anthophyllite (Timbrell et al, 1970).

1.3.2 The Surface Properties of Asbestos

The surface properties of asbestos fibres are governed by those ions found at the outer edge of the structure. These properties may modify the reaction of the fibre with commercially important chemicals, and also affect the interaction of the fibre with biological systems.

The surface of the chrysotile fibre comprises a layer of hydroxide ions bonded to an inner layer of magnesium ions. The hydroxide groups are negatively charged and thus impart an attractive force to ions of an opposite charge. This charge may be expressed in terms of the zeta potential, and a zeta potential of +100 mV for chrysotile in solutions of neutral pH has been recorded (Martinez and Zucker, 1960). The zeta potential may also depend upon the nature of any impurities, and brucite, for example, may contribute to the positive value of chrysotile (Chowdhury and Kitchener, 1975). Removal of the hydroxide and magnesium ions by acid leaching leads to exposure of the underlying surface, and results in negative value for the zeta potential (Chowdhury and Kitchener, 1975).

The outer surface of the amphibole fibre comprises a layer which is chemically similar to silica and thus relatively insoluble. The zeta potentials for amosite and crocidolite are -20 mV and -10 mV respetively, and this weak charge is completely due to the attraction of ions (Michaels and Chissick, 1979).

1.3.3 The Adsorptive Properties of Asbestos

The ability of a number of materials to adsorb onto the surface of asbestos fibres has been used to gain an estimate of the total surface area of the fibres. Early studies showed that chrysotile had a greater affinity for ammonia and water vapour than for nitrogen (Young and Healy, 1954). This result suggested that the polar surface of chrysotile has a greater affinity for polar molecules and

Table 1.2 Ranges of Trace Metal Contamination in Asbestos Samples From Different Regions

TYPE	Fe(%)	Cr(ppm)	Co(ppm)	Mn(ppm)	Ni(ppm)	Sc(ppm)
Chrysotile	0.6-48	317-1390	43-110	231-720	540-1820	0-12
Amosite	massive	31-35	7-11	11800-13350	33-100	0-5
Crocidolite	massive	16-20	0.4-10	140-880	8-100	0-0.6
Anthophyllite	2.0-4.4	584-870	24-50	986-1060	413-1360	0-5

After Morgan and Cralley (1973)

Weeks and Leineweber (1967) later confirmed that chrysotile does have a greater affinity for more polar organic compounds. In a solution of physiological pH, chrysotile can adsorb a greater quantity of negatively charged serum albumin than the amphiboles (Table 1.3) (Morgan, 1974), and all adsorption can occur within ten minutes. Leaching the chrysotile with acid reduced its adsorptive capacity for albumin (Morgan, 1974) because of the removal of the positively charged magnesium ions (Valerio et al, 1979).

These findings are of particular interest when considering the interaction of chemicals with fibres during the commercial treatment of asbestos, and also during biological interactions when the fibres may become coated with proteins or enzymes.

1.3.4 Chemical Reactivity of Asbestos

An examination of the reactivity of asbestos with various chemicals has shown that chrysotile is rapidly decomposed by strong acid, whereas the amphiboles are not; in addition all asbestos types exhibit a resistance to attack by alkalis (Speil and Leineweber, 1969) (Table 1.4). It has been shown that chrysotile intereacts readily with water and physiological solutions to produce magnesium ions and orthosilicic acid (Clark and Holt, 1960). Initially a high release of magnesium ions was observed, which declined to a steady level after several days; orthosilicic acid, however, was released slowly at first, and this increased to a higher steady rate after several days (Chowdhury, 1973). The amphiboles proved less reactive under similar conditions, and released only small quantities of cations (Chowdhury, 1973). It can therefore be concluded, that under biological conditions, and in particular over a long period of time in vivo, chrysotile fibres may readily dissolve, whereas the amphiboles may persist in a relatively intact form.

In order to facilitate the processing of asbestos, considerable use has been made of the chemical reactivity of chrysotile. Bundles of chrysotile fibres, if immersed in detergent solutions, will break down to form individual fibres; the amphiboles, however, do not respond in this manner (Hodgson, 1979). Processes have now been developed so that chrysotile can be wet-milled in the presence of soaps and fatty

Table 1.3 Adsorptive Capacity of Various Asbestos Types

SAMPLE		ALBUMIN ADSORBED (mg/g)
Chrysotile		
UICC Rhodesian	A	37
UICC Rhodesian	AR	15
UICC Canadian	B	45
UICC Canadian	BR	16
Canadian	A	55
Canadian	B	55
Canadian	D	55
Canadian	G	56
Canadian	H	64
Canadian	SFA	29
Amphiboles		
UICC Amosite		3.0
UICC Anthophyllite		8.5
UICC Cocidolite		4.9
Quartz	DQ_{12}	< 1

Samples AR and BR are milled versions of the original UICC samples.

SFA Chrysotile is a superfine milled version of Sample D.

Adapted from Morgan (1974)

acid sulphonates to produce fibrils with diameters of 200 - 500 A°; this wet-dispersed chrysotile may then be used in the production of asbestos yarn, papers and felts (Hodgson, 1979).

1.3.5 The Strength, Thermal Reactivity and Mechanical Disintegration of Asbestos

The various types of asbestos may exhibit different degrees of tensile strength; in general both chrysotile and crocidolite show a relatively high tensile strength, amosite and anthophyllite are intermediate, and tremolite and actinolite have proven relatively weak (Michaels and Chissick, 1979). The strength of all asbestos samples can be altered upon exposure to heat, and the strength may increase up to 250°C for amosite and crocidolite, and up to 550°C for chrysotile; at higher temperatures up to 800°C the strength decreases significantly (Burman, 1967). The loss of water from the amphiboles at temperatures up to 600°C corresponds with the decrease in fibre strength (Hodgson et al, 1965); above 800°C crocidolite breaks down to form silica and Na Fe-pyroxene, and amosite forms silica and Fe Mg-pyroxene. In the case of chrysotile, dehydroxylation occurs at 550°C which becomes complete at 800°C; above 825°C the amorphous fibres recrystallize to form forsterite (Mg_2SiO_4) and silica (Ball and Taylor, 1963; Brindley and Hayami, 1965). It is therefore accepted that whilst asbestos is not flammable, some alteration in its properties occur which may necessitate replacement of those materials exposed to high temperatures.

Asbestos may often be subjected to some mechanical disintegration during its utilization in automobile brake linings or its use as a reinforcing agent. Using X-ray diffraction techniques, it has been shown that the surface of chrysotile can easily be distorted during grinding (Harris, 1971), and this must be borne in mind when considering the potential pathogenicity of disintegrated fibres.

It is evident from the preceding sections that asbestos is in possession of a number of properties which are of considerable commercial use to man. The continued utilization of this mineral has therefore been ensured, in parallel with a persistent search for new mineralogical sources as well as substitutes for this fibre.

Table 1.4 Solubility of Asbestos Minerals in 25% Acid or Alkali

Percentage loss in weight after refluxing with acid or alkali for 2 hours

	HCl	CH_3COOH	H_3PO_4	H_2SO_4	NaOH
Chrysotile	56	23	56	56	1
Crocidolite	4	1	5	4	1
Amosite	13	3	12	12	7
Anthophyllite	3	1	3	3	1
Actinolite	20	13	21	21	9
Tremolite	5	2	5	5	2

Adapted from Speil and Leineweber (1969)

1.4 The Mineralogical Occurrence of Asbestos

The different types of asbestos have been found in various parts of the world, and these have already been extensively reviewed (Selikoff and Lee, 1978; Michaels and Chissick, 1979). In brief,serpentine, a rock comprising the mineral chrysotile, is mined in several parts of the world, namely Canada (Quebec, British Columbia and Yukon), U.S.S.R. (Urals and E. Siberia), and Africa (Rhodesia and Swaziland). Impure siliceous dolomite, another rock from which chrysotile may be obtained occurs in the Transvaal; chrysotile is also mined in Cyprus and in the U.S.A. (Vermont and Arizona), and accounts for approximately 95% of the world production of asbestos (Table 1.5). The asbestiform amphiboles have a more restricted occurrence and are obtained from those thermally metamorphosed, banded ironstones that are rich in magnesium and sodium. The main sources of crocidolite are Cape Province in S. Africa, Wittenoom Gorge in Australia, and Bolivia. Amosite is mined in the Transvaal, anthophyllite in Finland, tremolite in Italy, Pakistan and Korea, and actinolite in Taiwan.

1.5 The Mining, Milling and Manufacture of Asbestos

Asbestos is often mined by the opencast method (Walton, 1982) in which the underlying seam is exposed and the ore removed. Should the seam travel deeper underground, conventional mining techniques such as those used for coal production are employed. The ore is transported to mills where the fibres are released by crushing and milling. Moisture, impurities and foreign matter are removed, and the fibres separated into classified lengths (Walton, 1982). After this initial processing the asbestos can be stored in bags and transported to factories involved in the manufacturing industry. The manufacture of asbestos products does not necessarily occur in those countries in which asbestos is mined, and thus the fibre may be transported in bags to such countries as the U.K. for processing. Asbestos fibres have been used to manufacture a large number of products (Table 1.6), and up to 70% of the world's asbestos output is used for the production of asbestos cement (Clifton, 1972).

Any operation involving the mining, milling and manufacture of asbestos may result in the generation of airborne asbestos fibres.

Table 1.5 World Production Of Asbestos 1977

CHRYSOTILES	TONNE
U.S.S.R.	2,356,000
Canada	1,432,000
S. Africa	412,000
Europe	299,000
China	199,000
U.S.A.	95,000
S. America	72,000
Australia	68,000
Other countries	41,000
AMPHIBOLES	
S. Africa	
Amosite	67,000
Crocidolite	201,000

After Michaels and Chissick (1979)

Table 1.6 Asbestos Products - 1953

Raw Asbestos :

Yarn, felt, packing, paper, board, insulating wire, pipe covering, blocks, high temperature insulation, brake lining and brake blocks, fillers in plastics, flooring, pottery, asbestos cement, tiles, sheets, roofing, panels, insulating board, boiler insulation, paints, varnishes, sprayed asbestos, foundations to resist shock, filter fibres and pads, automobile undercoating.

After Selikoff and Lee (1978)

Such fibres present a considerable threat to asbestos workers as they may enter the body by a number of pathways (see Section 1.8). The asbestos industry has tried to overcome this by the use of water during the spinning and weaving of asbestos to reduce the fibre release (Lewinsohn et al, 1979). Areas of fibre contamination still exist, however, and some considerable importance has been attached to the identification and quantitation of fibres contaminating the working environment.

1.6 The Identification of Asbestos Fibres

Asbestos fibres suspended in the ambient air may be collected by passing the air through a filter head and collecting the fibres on a 0.8μm pore filter (Ayer et al, 1965). The filter can be made transparent by using an appropriate solvent, and the fibres examined using a Phase Contrast microscope (A.R.C., 1971). The asbestos type can be identified using a polarizing microscope, and is dependent upon the pleochroism and refractive index values of the fibres (Selikoff and Lee, 1978). Fibres may also be examined using both scanning and transmission electron microscopy techniques (Section 2.1.1-2.1.3), and the type identified using X-ray powder diffraction patterns or energy dispersive X-ray analysis techniques. A threshold limit value of 0.5 fibres, 0.2 fibres and 2 fibres per cm^3 of air for amosite, crocidolite and chrysotile respectively has been established (Health and Safety Executive 1981), and these figures are always under constant review.

1.7 Sources of Human Exposure to Asbestos Dust

Those occupations that involve the mining, milling, manufacture and use of asbestos products can result in the disruption of the mineral and release of airborne fibres, thereby presenting a hazard to the exposed individual (Becklake, 1976). In addition to occupational exposure, a number of non-occupational sources of asbestos contact have been shown. Neighbourhood exposure from mines and mills can occur (Wagner et al, 1960), and also domestic exposure from workers taking home dusty overalls (Champion, 1971). The erosion of building materials may produce airborne fibres (Sebastien , 1976), and agricultural workers may be exposed

to naturally occurring asbestos fibres from the soil (Burilkov and Michailova, 1970). Some asbestos contamination of filtered wines and beverages, the tap water of a number of Canadian cities (Cunningham and Pontefract, 1971) and drugs for oral and parenteral use (Selikoff and Lee, 1978) has been recorded.

1.8 Sites of Entry of Fibres Into the Body.

There are a number of routes by which the contaminating asbestos fibres may enter the body, and the two major entrances are the lung and the gastro-intestinal tract.

1.8.1. The Lung.

Particles inspired from the atmosphere must first pass through the nose, where those particles larger than 10 μm. diameter are filtered out by hairs lining the nostrils (Proctor, 1966). The trapped particles may then be passed in a mucous layer, along the ciliated epithelium which lines the nostrils, and towards the pharynx. Under such conditions as nasal blockage, or during heavy exercise or work, the individual may resort to mouth breathing thereby allowing the larger particles access to the thoracic airways (Becklake, 1976).

The thoracic airways comprise the cartilaginous trachea which divides at the base to give two bronchi, and each bronchus sub-divides approximately twenty times into lobar, segmental terminal and respiratory bronchioles. With each successive division there is a decrease in airway diameter, but an increase in total cross sectional area; this leads to a decrease in the velocity of the inspired air as the smaller airways are reached (West, 1976).

The inhalation of airborne asbestos fibres is determined not only by the structure of the lungs, but also by the aerodynamic properties of the individual fibres themselves, and consideration of these properties is of considerable importance.

1.8.2 The Aerodynamic Properties of Inhaled Fibres in Relation to the Lung.

A fibre for the purpose of this thesis is defined as a particle whose length:diameter ratio is greater than 3:1. The tendency of a fibre to be deposited in the lung is governed by its "free-falling speed", this is proportional to the square of the particle diameter (Timbrell, 1965), and is also dependent upon the size and force of each inspiration. When a particle or fibre contacts an airway or alveolus it does not become airborne again, and thus is termed "deposited" (Parkes, 1974).

There are five physical processes influencing the probability of deposition in the lung, and these comprise:

a) Inertial Impaction: When the direction of flow of the airstream entering the airways is changed by the branching and curving of the bronchioles, the fibres are carried by their own inertia along their original path and consequently deposit on the walls. The probability of deposition occurring is proportional to the velocity of the airflow, density and diameter (squared) of the particle, the angle of branching, and inversely proportional to the diameter of the airway. This is the major process by which the larger particles are arrested in the airways (Landahl, 1963), particularly at the bifurcations.

b) Sedimentation: The sedimentation of a particle is determined by its density and diameter (squared), and the probability of sedimentation occurring is increased by the slope and narrowness of the airway concerned. This gravitational settlement of the fibres occurs in the large airways and allows fibres of less than 3 μm diameter only to pass into the small airways (Timbrell, 1970); deposition by sedimentation is also important in the small airways and alveoli.

c) Diffusion: Diffusion is an important mechanism for deposition of particles with diameters less than 0.5 μm and occurs mainly in the small airways, alveolar spaces, and upon the tracheal wall (Landahl, 1963).

d) Interception: Interception occurs when particles of irregular shape such as fibrous asbestos and in particular curly chrysotile align closely enough to the bronchiolar wall to touch the surface. The probability of interception occurring is greater at bifurcations and in narrow airways (Selikoff and Lee, 1978).

e) Electrostatic Precipitation: An interaction of electrostatic forces may occur between small, charged fibres and the alveolar wall, resulting in electrostatic precipitation (Selikoff and Lee, 1978).

In summary, work by Harris and Fraser (1976) and Timbrell (1965) suggests that the largest and longest particles would be deposited by interception and impaction in the large airways and at bifurcations. Particles with diameters greater than 0.25 μm deposit by sedimentation, and long particles by interception in the small airways. Particles with diameters less than 0.25 μm deposit in the bronchioles and alveoli by sedimentation, diffusion and electrostatic precipitation.

The pattern of breathing may also alter deposition in the lung, it is expected that increased inspiration during heavy physical effort would result in a greater deposition of particles (Lippmann and Albert, 1969).

1.8.3 Other Sites of Entry of Asbestos

The gastrointestinal tract is a source of entry for contaminating asbestos, and it is known that fibres can enter the alimentary canal via a contaminated water supply (Speil, 1974), beverages filtered through asbestos pads (Cunningham and Pontefract, 1971), or powders and pills in which talc is used. Fibres may also be directly swallowed from the atmosphere in dusty working conditions, or alternatively from the pulmonary clearance system.

It has been suggested that talc contaminated by asbestos may enter the uterus, and the anus may permit entry of asbestos from dusty undergarments (Selikoff and Lee, 1978). Some asbestos may also be introduced under the surface of the skin in injection mixtures which have previously been filtered through asbestos pads (Nicholson et al, 1972).

1.9 Clearance and Transport of Asbestos to Target Sites in the Body.

When the fibres have entered and been deposited in the body their fate is decided by a number of mechanisms which may either clear the fibres completely, or allow their transport to various sites in the body.

1.9.1. Clearance of Fibres From the Body.

Inhaled particles that have become deposited on the walls of the bronchi and bronchioles may be removed from the lung by passage along the mucociliary escalator. Particles depositing in the non-ciliated airways and alveoli may be ingested by alveolar macrophages and transported to the mucociliary escalator (Brain and Valberg, 1974), and this clearance mechanism is believed to be 98% efficient (Gross and DeTreville , 1972). Additional throat clearing and coughing can assist the passage of larger quantities of mucus from the upper airways in such diseases as bronchitis and during the ciliastasis which occurs as a result of cigarette smoking (Albert et al, 1973). Pulmonary and nasal mucus mix at the pharynx and are generally swallowed or expectorated (Evans et al, 1973). The clearance of fibres through the gut has been demonstrated by Evans et al (1973) who used radioactively labelled crocidolite in rat inhalation studies, and showed that 75% of inhaled fibres are eventually excreted in the faeces. It has also been suggested that fibres may be excreted in the urine of those persons ingesting amphibole contaminated water (Selikoff and Lee, 1978).

1.9.2. The Macrophage and the Ferruginous Body.

The development of the "ferruginous body" may be a means of protecting the lung from those fibres longer than 5μm that cannot be ingested completely by one macrophage (Davis, 1970). Its development has been described in electron microscope studies by Davis (1970) and Suzuki and Churg (1969a and b). Studies concerning the development of the ferruginous body have shown that several macrophages may attempt to engulf a long fibre and become fused; during the process of fusion the fibre becomes coated first with

acid mucopolysaccharide, and then by iron micelles (Suzuki and Churg, 1969a and b; Davis, 1970). Ferruginous body formation is thought to be an on-going process, and uncoated fibres may become coated several months after installation (Davis, 1970).

The majority of ferruginous bodies are formed on amphibole fibres (Langer et al, 1971), but only on those fibres of chrysotile that are straight and over several μm in length; this may be due to the fibres dissolving in biological conditions before the ferruginous body can form. Ferruginous bodies, as well as being found in the lungs of occupationally exposed persons have also been found in the lungs of non-occupationally exposed city dwellers (Langer et al, 1970). The bodies may also form on other, non-asbestiform particles (Gross et al, 1969); and have been observed in tissues other than the lung, although it is not known whether they were created in situ or transported to this position (Selikoff and Lee, 1978).

1.9.3 Transport of Fibres Around the Body.

The passage of fibres from their original site of deposition to an area where the initiation of disease may occur is still a subject of controversy. Using a transmission electron microscope to search for fibres in human tissue, asbestos has been identified in lung parenchyma, bronchial tissue, lymph nodes, parietal pleura, pleural fluid, peritoneum, liver, stomach, bowel wall and colon (Bignon et al, 1979).

Inhaled particles were originally thought to be transported from the alveolus to the interstitium by alveolar macrophages (Brain et al, 1977). However, recent work by Brody et al (1981) showed that fibres may be passed via Type I epithelial cells, through the basement membrane and thus enter the interstitial cells (i.e. fibroblasts and macrophages). They may also be carried to the lymphatics and hilar and mediastinal lymph nodes (Gross et al, 1973), and inhaled fibres may be transported to the parietal pleura via the lymphatic system (Taskinen et al, 1973).

It has been suggested that some fibres may penetrate the bronchial wall, thus causing metaplasia (Selikoff and Lee, 1978), and studies by Mossman and Craighead (1976) have demonstrated, using tracheal organ cultures, that fibres can be transported to the tracheal submucosa within seven days.

Evidence for the transport of asbestos across the gut wall into the peritoneal cavity is scanty. Studies by Pontefract and Cunningham (1973), who used massive doses of asbestos, showed that asbestos fibres can penetrate the gut wall. However, ingestion experiments by Webster (1974) and Smith et al (1965) showed neither carcinoma of intestinal epithelium or peritoneal mesothelioma; Bolton et al (1982b), in a detailed study of prolonged asbestos ingestion in rats, found no evidence of fibre penetration of the gut.

The final deposition of fibres at various target sites in the body, leaves the mineral freely available to interact with a number of biological systems, thereby resulting in the manifestation of a variety of diseases.

1.10 Epidemiological Evidence for and Pathological Manifestations of Asbestos-Induced Disease.

As early as 61 to 114 A.D. Pliny the Younger commented on the sickness of slaves who worked with asbestos (Selikoff and Lee, 1978); however it was not until 1907 that lung disease was linked with asbestos exposure in Britain (Murray, 1907). It is now generally accepted that asbestos can initiate a number of pathological events such as asbestosis, bronchial carcinoma, pleural and peritoneal mesothelioma, pleural plaques, gut, ovarian and breast tumours and also skin granulomas and some immunological changes (Harington et al, 1975).

1.10.1 Asbestosis.

Asbestosis is defined as a pulmonary fibrosis due to inhalation of asbestos. Auribault in 1890 (cited by Lynch et al, 1935) initially suggested a correlation between the severe pulmonary fibrosis occuring in French textile workers and their exposure to asbestos; this

was later supported by Murray (1907) and Merewether and Price (1930). More recent surveys have shown an increased incidence of pulmonary fibrosis in populations exposed to asbestos in mines and mills (McDonald et al, 1971), dockyards (Sheers and Templeton, 1968) and also non-occupationally exposed groups living in the vicinity of mines and mills (Webster, 1964).

Asbestosis is one of the pneumoconioses and is typified by diffuse interstitial fibrosis which tends to progress and is incurable. The pathological features of this disease have been described by Hourihane and McCaughey (1966), and can range from small localised areas of fibrosis to diffuse fibrosis affecting both lungs. The lower lobes tend to be affected first, with gradual spread to the upper lobes. On occasions emphysema can occur in association with asbestosis; also massive progressive fibrosis when exposure to mixed dust such as silica has also occurred (Solomon et al, 1971). The early stages of development of fibrosis can often be seen at the respiratory bronchioles where macrophages and dust particles collect, initiating peri-bronchiolar fibrosis which may later develop outwards causing interstitial fibrosis (Parkes, 1973).

The most characteristic clinical features of asbestosis are crepitations and cough, with dyspnoea as the disease progresses; some finger clubbing has also been observed (Parkes, 1973). Radiologically, the disease is characterised by small irregular and/or rounded opacities (Becklake, 1976).

1.10.2 Carcinoma of the Lung.

An association between asbestos and lung cancer was originally suggested in the 1930s (Becklake, 1976) and confirmed by Gloyne in 1951. More recently carcinoma of the lung has been found to occur in conjunction with asbestosis (Jacobs and Anspach, 1965), and a link between carcinoma, asbestos and cigarette smoking has led research workers to propose a synergism between asbestos and cigarettes (Berry et al, 1972). The tumour usually occurs in areas of fibrosis and often in the lower lobes of the lung (Jacobs and Anspach, 1965).

Pleural effusion has also been associated with asbestos exposure, it usually occurs in association with some chest pain, and the fluid is often blood-stained. As yet it is not known whether the fluid precedes the occurence of lung carcinoma or mesothelioma (Chahinian et al, 1973).

1.10.3 Mesothelioma of the Pleura and Peritoneum.

Mesothelioma is an incurable malignant tumour of the linings of the pleural or abdominal cavity, and can occur 20 to 40 years after initial exposure. The occurence of mesothelioma was regarded as relatively rare i.e. one per 1,000,000 per year in the general population (Becklake, 1976) until the 1940s when Wyers noted the association of the tumour with asbestosis (Parkes, 1974). In 1960 Wagner reported an increased incidence of mesothelioma in people exposed to crocidolite either occupationally or non-occupationally in S. Africa. Later studies confirmed an increased mortality due to mesothelioma in factory workers (Newhouse, 1973), textile workers (Selikoff and Lee, 1978) and shipyard workers (Edge, 1976).

The mesothelioma is said to initiate primarily from the mesothelial cells and can be present in four forms which are characterised according to their microscopic appearance: tubopapillary, sarcomatous, undifferentiated, polygonal and sometimes mixed (Parkes, 1974). Both pleural and peritoneal tumours tend to be lobular and, particularly in the case of peritoneal mesothelioma, a glutinous ascitic fluid is often seen. The clinical characteristics of this disease are chest pain, breathlessness and weight loss in the case of pleural mesothelioma; and pain, swelling and weight loss in peritoneal mesothelioma (Becklake, 1976).

1.10.4 Pleural Plaques.

Pleural plaques consist of a fibrotic thickening of the parietal pleura and can be hyaline or calcified. An examination of ashed tissue has shown the presence of fibres in association with pleural plaques (Hourihane et al, 1966). Epidemiological data indicates an increase in the incidence of pleural plaques in dockyard workers (Sheers and Templeton, 1968), insulation workers (Selikoff, 1965) and also agricultural workers exposed to asbestos fibres present in the soil (Burilkof and Michailova, 1970).

Pleural plaques occur as discrete, grey-white lesions on the inner surface of the rib-cage and diaphragm (Becklake, 1976) and consist of collagenous connective tissue. Calcium deposition is seen in some plaques and occurs as granules along the collagen fibres (Meurman, 1966). Pleural plaques alone appear to have little effect on the respiratory system apart from a small reduction in lung volume (Becklake, 1976).

1.10.5 Other Extrathoracic Effects of Asbestos.

There is some epidemiological evidence to suggest that an increased incidence of carcinoma of the gastro-intestinal tract is associated with exposure to asbestos (Selikoff and Lee, 1978), and some fibres have been found in association with stomach tumours (Henderson et al, 1975). Asbestos exposure has also been linked with cancer of the larynx (Stell and McGill, 1973; Newhouse and Berry, 1973), although Morgan and Shettigara (1976) suggest that the risk of laryngeal cancer is confined to those who smoke. Links between asbestos exposure and breast cancer (Doniath et al, 1975) and also ovarian cancer (Graham et al, 1967; Henderson et al, 1971) have been suggested, although the role of asbestos in the induction of these cancers is not yet understood.

An association between asbestos exposure and the malignancies of the haematopoietic system has been suggested (Preger, 1978). These include lymphosarcoma, multiple myeloma, polycythemia vera and Waldenstrom's macroglobulinanaemia (Lieben, 1966; Gerber, 1970). It is also known that asbestos can induce warts or granulomas on the skin upon penetration of the epidermal tissue (Selikoff and Lee, 1978).

1.10.6 Asbestos-Induced Alterations in the Immune System.

Evidence concerning the effects of asbestos on the immune system is scanty, although the recently heightened awareness of the importance of any immunological defects has resulted in an increase in the number of groups of research workers examining this problem. An association between asbestosis and autoimmune disease has been observed (Rickards and Barrets, 1958), together with an increased

incidence of rheumatoid factor (Pernis et al, 1965), antibodies in sera (Turner-Warwick and Parkes, 1970) and hyperimmunoglobulinaemia (Lange et al, 1974). Some alterations in cell-mediated immunity of asbestosis patients has also been noted, including a marked decrease in T-lyphocytes (Kang et al, 1974; Kagan et al, 1977; Ramachander, 1975), and a diminished functioning of suppressor cell activity (Gaumer et al, 1981).

From the preceding sections it is evident that a number of disease states have been associated with exposure to asbestos fibres, and asbestosis and cancer are the best documented of these. A number of epidemiological studies have been carried out to try and identify those physicochemical properties of asbestos which have the potential to initiate disease.

1.11 Epidemiological Evidence for Association of Fibre Type, Dimension and Dose of Asbestos with Asbestosis and Mesothelioma.

Epidemiological evidence is available to suggest that all of the commercially available asbestos fibre types are capable of initiating asbestosis and lung cancer in humans; and all, with the exception of anthophyllite (Meurman et al, 1974), show an increased association with mesothelioma (Selikoff and Lee, 1978). The suggestion that some asbestos types may be more fibrogenic/tumourgenic than others has led to consideration of the dose to which the subjects have been exposed in order to try and evaluate a threshold value for initiation of a pathological reaction. Studies would suggest that fibre type, dimensions and dose must be taken into consideration.

1.11.1 Fibre Type.

Crocidolite has for a number of years been considered the most hazardous of the asbestos types, and this is primarily due to its early association with a high mesothelioma incidence in the North Western Cape Province of S. Africa (Wagner et al, 1960). Further support for this view came from studies by Enterline and Henderson (1973) and Weill et al (1975) in which lung disease in workers exposed to either crocidolite and chrysotile together, or to chrysotile alone

were compared. A greater reduction in lung function and a greater number of deaths was observed for those groups of workers exposed to chrysotile and crocidolite together rather than to chrysotile alone. Epidemiological studies in which workers were exposed to chrysotile alone have suggested that this dust is the least pathogenic of the commercially available types. Studies by Braun and Truan (1958) and McDonald et al 1974) show the incidence of lung cancer in workers exposed to chrysotile to be five times above the expected value, and for amosite ten times above the expected value (Selikoff et al, 1972). Becklake (1976) and Kleinfield (1973) suggest that the order of ability of the fibre types to induce mesothelioma is crocidolite>amosite>chrysotile> anthophyllite. Timbrell (1973) suggested that the curly nature of the chrysotile fibres may hinder their ability to enter the lung and to transmigrate within the lung parenchyma. Certainly, crocidolite is thought to be more fibrogenic than chrysotile (Becklake, 1976), although there is little epidemiological data available for the asbestos types tremolite and actinolite, to be able to assess their relative degrees of human pathogenicity (Becklake, 1976).

1.11.2 Fibre Dimensions.

Some attempts have been made to explain the varying pathogenicity of the different asbestos types in terms of their respective fibre dimensions. Crocidolite mined in the Transvaal was found to induce a lower number of mesotheliomas than crocidolite mined in the North Western Cape (Wagner et al, 1960; Sluis-Cremer, 1970). This observation was discussed by Timbrell et al (1971) who showed that the North Western Cape fibres are shorter and finer than those from the Transvaal, and he suggested that the Cape fibres may avoid interception in the airways, thereby leading to an increase in the number of fibres entering the lung. Amosite has been shown to induce more mesotheliomas in the manufacturing than the mining industry (Becklake, 1976), and this had been attributed to the smaller size available for inhalation during the manufacturing process. Unfortunately the majority of epidemiological studies have involved assessment of fibre exposure using the light microscope. Many of

the fibres present in an asbestos population are below the resolution of the optical microscope, and it can therefore be said that only the exposure of man to fibres of greater than 0.4μm diameter has been assessed (Cooper, 1978).

1.11.3 Fibre Dose.

In order to explain the variation in biological response of man to inhaled dust, Hatch (1968) introduced his three-dimensional concept of a dose relationship of response to stimulus. This concept suggests that a given dose-response curve can be drawn for an individual person, but the curve can only be applied to someone of the same "susceptibility". Susceptibility involves a consideration of all biological features of the lung, the fitness of the person and also the immunological susceptibility. Unfortunately when using this model, it is only possible to estimate the dose inhaled by the person, as there is no practical means available for measurement of the clearance and retention of occupationally inhaled dust.

The measurement of dose in epidemiological studies has generally been expressed in terms of number of years' service in industry, total years of exposure, or estimates of dustiness of job (Becklake, 1976). Unfortunately, the long latent period observed between the initial exposure to asbestos and the onset of disease usually entails some loss of data concerning dust type and concentration etc. Several studies have indicated that there is a relationship between dose and lung cancer (Selikoff, 1967; Liddell et al , 1977). Berry (1977) has correlated the risk of contracting lung cancer with degree of exposure to asbestos, taking into account the non-occupational background incidence of lung cancer. He concludes that whilst current exposure standards might remove excess lung cancer, there may still be an effect.

Ashcroft and Heppleston (1973) and Sebastien et al (1975) examined the asbestos content of fibrosed tissue, and have shown a progressive increase in asbestos concentration which paralleled the increased degree of asbestosis. The occurrence of mesothelioma

does not appear to be connected with the dose level, and evidence for mesothelioma induction after only brief neighbourhood exposure has been reported (Wagner et al, 1960; Champion,1971).

1.11.4 Synergism and Cancer.

Acheson and Gardener (1979) have suggested that a synergism may occur between the amphiboles and chrysotile, as mixtures of these two asbestos types appear most commonly in the lungs of mesothelioma patients than either type of fibre alone. More importantly however, considerable epidemiological data is available to indicate that smoking greatly enhances the risk of lung cancer in asbestos workers (Hammond and Selikoff, 1973; Hammond et al, 1979) and a synergistic effect between these two agents has been suggested.

The currently available epidemiological data has certainly demonstrated that the various asbestos types may have different fibrogenic and carcinogenic potentials, and this may possibly be related to the dimensions of the fibres. In order to try and elucidate the situation, many workers have turned to the use of animal models in which defined asbestos types of known dose and dimension can be applied to a specific area of the body.

1.12 Experimental Data from Animal Studies Concerning the Carcinogenicity and Fibrogenicity of Asbestos Fibres.

A number of animal models have been employed to examine the in vivo effects of asbestos fibres, and these have mainly involved the use of rats, and occasionally mice, guinea pigs and hamsters. It must be borne in mind however, when interpreting data from such experiments, that the dimensions of the animal airways differ considerably from those of the human, the life span is shorter, and there may be some immunological differences. A large number of experiments have been reported in which the in vivo effects of asbestos have been related to the physicochemical properties, the dosing regime, and synergism of the fibres; only a few of the more pertinent references will be discussed here.

1.12.1 Fibre Type, Surface Properties and Synergism.

Asbestos will produce fibrosis and tumours in animals whether the route of administration is intratracheal or intraperitoneal (Bignon et al, 1979). Inhalation studies in rats have shown that all asbestos types can initiate lung fibrosis, although Wagner et al (1974) reported that amosite was less fibrogenic than other types of asbestos, and Davis et al (1978) reported that chrysotile was more fibrogenic towards the rat lung than crocidolite or amosite following exposure to inhalation clouds of equal fibre number. In general, fibrosis can still increase even after environmental exposure to dust has ceased (Wagner et al, 1974). Attempts at preventing asbestosis in animals by using polyvinyl-pyridine-n-oxide (PVPNO), a known antisilicotic agent (Schlipköter and Beck, 1965), were unsuccessful , thereby suggesting that asbestos may interact with the cell membrane and initiate fibrosis via a different mechanism from quartz.

Bronchogenic carcinoma has been induced in rats by chrysotile, crocidolite, amosite and anthophyllite (Wagner et al, 1974), and the tumour incidence showed a tendency to increase as the time of exposure increased (Wagner et al, 1974). Studies using the technique of intrapleural instillation of asbestos into rats have demonstrated that all types of asbestos and also a brucite sample can induce mesotheliomas; the tumour induction was dose dependent (Wagner and Berry, 1969; Wagner et al, 1973). It has been reported following an inhalation study that an exposure time of one day is sufficient for a number of mesotheliomas to occur (Wagner et al, 1974). A number of studies have examined the carcinogenicity of chrysotile following alteration of the physocochemical properties of the sample. It was reported that heating chrysotile to 850°C effectively reduced its ability to induce mesotheliomas by 50% (Bolton et al, 1982a), and leaching chrysotile in hydrochloric acid also reduced its carcinogenicity (Morgan et al, 1977; Lafuma et al, 1980; Monchaux et al, 1981). It must, however, be borne in mind that such a stringent treatment of chrysotile may not only modify the surface properties but also the ability of the sample to decompose in vivo,

and this situation is still to be clarified.

The possibility that asbestos may act synergistically with other carcinogens has attracted some interest. Harington (1962) suggested that the contaminating oils and waxes on asbestos fibres may prove carcinogenic, Wagner (1969, 1973) however, later reported that fibres from which the oils had been removed were as carcinogenic as the natural fibre. An increase in the number of neoplasms was observed when chrysotile asbestos was intratracheally instilled into animals in combination with benzo(a)-pyrene, a cigarette smoke component, (Smith et al, 1968: Shabad et al, 1974), and this is consistent with epidemiological evidence concerning the synergism between smoking and asbestos exposure and the occurrence of bronchial carcinoma (Hammond and Selikoff, 1973; Hammond et al, 1979). Asbestos may also potentiate dimethylbenz(a)anthracene-induced rat tracheal carcinomas (Topping and Nettesheim, 1980) and also oncogenesis by Moloney murine sarcoma virus in mice (Kanazawa et al, 1979); it has been proposed that asbestos may act as a tumour promoter (Topping and Nettesheim, 1980).

1.12.2 Fibre Dimensions

All of the commercially used asbestos types have been associated with disease; as they all differ in their respective chemical compositions, it has been considered for a number of years that fibre dimensions may play an important role in determining the development of fibrosis, carcinoma and mesothelioma. Vorwald et al (1951) initially reported that animals which had inhaled chrysotile fibres of 20 to 50 μm length developed more pulmonary fibrosis than those inhaling fibres of less than 3μm length. Using both intratracheal and intraperitoneal routes of administration, Klosterkötter (1968) demonstrated that ball-milled chrysotile and crocidolite initiated less fibrosis than the parent samples. Similar results have been reported in rats exposed to long and short fibre amosite (Timbrell and Skidmore, 1968), monkeys treated with ground crocidolite (Webster, 1970), and mice inoculated

intrapleurally with either ground chrysotile or its parent (Davis 1972). In an inhalation study Davis et al (1978) demonstrated that chrysotile was more fibrogenic than crocidolite. It was suggested that this increased fibrogenicity may have been due to the presence of a greater number of fibres longer than 20μm in the chrysotile cloud. Wright and Kuschner (1977) examined the ability of crocidolite and glass fibre of known dimensions to induce lung fibrosis in guinea pigs; they concluded that fibres longer than 10μm cause fibrosis, whereas those shorter than 10μm do not.

With regard to the carcinogenic potential of asbestos, both short fibre crocidolite and chrysotile proved less carcinogenic than the long fibre samples (Stanton and Wrench, 1972; Smith et al, 1972), and Maroudas et al (1973) suggested that those fibres of more than 20μm in length and less than 2.5μm diameter would be carcinogenic. The effect of fibre size on sarcoma production was examined extensively by Stanton (Stanton et al, 1977; Stanton and Layard, 1978) who implanted fibrous samples of known fibre length distribution into the pleural spaces of rats. It was concluded that the carcinogenicity of a sample may depend upon its dimensional configuration, and a close correlation between pleural sarcoma induction and numbers of fibres longer than 8μm in length and less than 1.5μm diameter was noted (Stanton and Layard, 1978).

There is undoubtedly a quantity of data available which would firmly suggest a role for the dimension of a fibre in determining its pathogenicity. Pott (1978) has concluded that chemical composition is important only in terms of determining the durability of the sample in vivo; and he suggests that there is a gradual transition in degree of pathogenicity rather than a demarcation between sizes. Presumably future animal studies using defined fibre populations, in conjunction with in vitro experiments to determine the cell at risk, will further elucidate this situation.

The use of an animal model is the obvious choice for experiments designed to investigate the pathogenicity of asbestos. It has, however become evident that not only is this type of system expensive, but it generally involves waiting for a long period of time to obtain a result, for example it may take a year or more for fibrosis or cancer to develop following exposure to asbestos. A number of research groups have turned to using tissue culture systems, as these have the advantage of being fairly cheap in cost and a predictive result may be obtained relatively rapidly. Most importantly, the the tissue culture system permits a close observation of the direct effects of asbestos on those cells which may be potential targets for interaction with fibres *in vivo*; it may therefore be possible to predict from results obtained during *in vitro* experiments the mechanism of action or degree of pathogenicity of asbestos *in vivo*.

1.13 Experimental Data from Tissue Culture Systems Regarding the In Vitro Effects of Asbestos Fibres.

A large number of experiments have been carried out to investigate the direct effects of asbestos on potential target tissues from the body. These have involved an examination of whole tissue explants from the lung, trachea and pleura, as well as a number of primary cells such as the fibroblast, mesothelial cell and macrophage. A number of research groups have also discovered the advantages of permanent cell lines, these being the ready accessibility of the cells and also their reproducibility when used in experimental model systems. This has resulted in the generation of a considerable body of data relating to the *in vitro* effects of asbestos fibres, and a number of the currently used tissue culture systems will be discussed in the following sections.

1.13.1 Organ Culture Systems.

The most relevant organs from which explants have been taken for the examination of the direct effects of asbestos have comprised the lung, pleura and trachea. Davis (1967) was one of the first research workers to examine the effects of chrysotile fibres of less than 5μm in length on cultured guinea pig lung explants. His main concern was with alveolar macrophage population, which altered in

morphological appearance following treatment with dust, and it was noted that the intra-lysosomal content of the cells increased. Later reports by Rajan et al (1972) and Rajan and Evans (1973) described the response of the embryonic lung and human adult pleura to treatment with crocidolite. A proliferation of both fibroblasts and mesothelial cells was noted, as well as an enhanced release of lysosomal enzymes.

Mossman has extensively examined the response of cultured hamster trachea to asbestos (Mossman et al, 1977; Mossman and Craighead, 1981). Both crocidolite and chrysotile proved cytotoxic towards the differentiated respiratory mucosa, and some sloughing of the mucosal layer was observed. Seven days following treatment both basal cell hyperplasia and squamous cell metaplasia were seen. In a similar study Frank (1980) confirmed that both amosite and crocidolite can induce basal cell hyperplasia in hamster trachea. Mossman and Craighead (1981) also suggested that asbestos may prove synergistic with cigarette smoke by carrying adsorbed polycyclichydrocarbons into the basal cells; some metabolic stimulation may occur, thereby enhancing the susceptibility of the basal cell to the action of the carcinogen.

1.13.2 The Mesothelial Cell.

It is now generally accepted that the mesothelial cell is a likely candidate for carcinogenic transformation by asbestos, and a French research group is currently examining the effect of asbestos on this cell type. Thiollet et al (1978) developed a method of isolating and culturing mesothelial cells from rat parietal pleura, and Jaurand et al (1979a) demonstrated that these cells were actually capable of phagocytosing chrysotile fibres. Phagocytosis of chrysotile did not, however, result in any alteration in chromosome morphology, as assessed by sister chromatid exchange induction (Kaplan et al, 1980b), although an increased doubling time of the exposed cultures was noted (Kaplan et al, 1980a), which would suggest that asbestos does have an effect on the metabolic functioning of this cell.

1.13.3 The Fibroblast.

Asbestos is a fibrogenic agent, and this has stimulated the establishment of a number of studies to investigate the direct effect of asbestos on fibroblasts in vitro. The effect of chrysotile on cultured lung fibroblasts has been extensively examined by Richards et al (1971, 1973, 1976); an initial cytotoxic effect was observed followed by renewed growth and cell recovery, although some deposition of reticulin fibres and an alteration in the glycosaminoglycan ratio (hyaluronic acid:chondroitin sulphate) released was noted.

Heppleston and Styles (1967) were the first to demonstrate that macrophages, upon phagocytosis of silica particles, may release a fibrogenic factor which is capable of initiating fibroblastic synthesis of collagen; later studies by Aalto and Kulonen (1979) and Aalto et al (1979) have shown that both silica and chrysotile can stimulate macrophages to release a fibrogenic factor. Bateman et al (1980) examined this further by implanting diffusion chambers in the peritoneal cavities of mice. The chambers contained macrophages and asbestos, and a significant fibrotic response was observed which would suggest that macrophages may release an enzyme or factor which either acts directly upon fibroblasts, or upon other macrophages which then produce the fibrogenic factor.

Bearing in mind the reports that there is epidemiological evidence available to suggest a synergism between asbestos and polycyclic aromatic hydrocarbons (PAH) from cigarettes, Daniel et al (1980) examined the effect of chrysotile on PAH metabolism in cultured fibroblasts. They reported a increased cell death and DNA binding of activated benzo(a)pyrene (BP) when the cells were exposed to chrysotile 24 hours prior to addition of BP. Brookes & Lawley (1964) have shown that there is a relationship between DNA binding and the carcinogenic potential of PAH and the data from the work of Daniel et al (1980) would lend support to the theory of synergism between PAH and asbestos.

There are a few reports available concerning the cytogenetic effects of asbestos on fibroblasts. Chrysotile and crocidolite have the ability to induce chromosome abnormalities in hamster fibroblasts (Sincock and Seabright, 1975; Lavappa et al, 1975), and crocidolite and amosite may increase the sister chromatid exchange rate in hamster ovarian fibroblasts (Livingston et al, 1980). Asbestos may therefore disturb the chromosomal stability of the fibroblast, but the long term manifestation of this effect is, as yet, undecided.

1.13.4 The Erythrocyte.

The initial observation by Dognon and Simonot (1951) that silica particles have the ability to lyse erythrocytes, has prompted considerable use of this cell type as a model by which direct membrane/dust interactions can be examined. In particular, the simplistic nature of this system, which relies on an assessment of the haemoglobin released from erythrocytes as a measure of cell damage, has encouraged its potential use as a predictor of in vivo pathogenicity.

Silica (SiO_2) may be obtained as a respirable particulate, available in a number of pure and impure states, and, like asbestos, is well-known for its fibrogenic action in vivo (Harington et al, 1975). Thus several investigators have examined and compared the haemolytic activities of both asbestos and silica to see if the mechanisms by which they cause membrane damage are similar (Harington, 1976). Following the work of Dognon and Simonot (1951), a number of other investigators confirmed the haemolytic action of silica (Stalder and Stöber, 1965; Macnab and Harington, 1967). Macnab and Harington (1967) also demonstrated that chrysotile was a potent haemolytic agent, whereas the known fibrogenic agents, crocidolite and amosite (Vigliani, 1968) did not show such a great degree of haemolytic activity. This difference in the haemolytic ability of the various types of asbestos has been demonstrated by several workers (Schlipköter, 1968; Schnitzer and Pundsack, 1970). Harington et al (1971b) also showed that the haemolytic ability of actinolite could vary depending upon its source; that anthophyllite was a strong lytic agent; and that brucite ($Mg(OH)_2$) caused a greater degree of haemolysis than chrysotile. A later study by Hefner and Gehring (1975) showed that the haemolytic activity of a particulate dust

correlated with its fibrogenic ability *in vivo*, and thus the rate of haemoglobin release over a period of time could be used as a measure of the fibrogenicity of a sample.

A number of mechanisms have been proposed to explain the haemolytic nature of silica and chrysotile, as well as the apparent low reactivity of the amphiboles, and these will be discussed in the following sections.

1.13.4.1 The Haemolytic Activity of Silica.

A number of theories have been proposed for the mechanism by which silica causes haemolysis. Nash *et al* (1966) proposed that silica toxicity was caused by hydrogen-bonding between silicic acid on the surface of the particle and the phosphate groups of phospholipids and amide groups of proteins on the cell membrane. They provided support for this hydrogen-bonding theory by demonstrating the protective effect of polyvinyl-pyridine-N-oxide (PVPNO), an effect also reported by Stalder and Stöber (1965). It was postulated that the polymer PVPNO, which has a high surface charge, could form strong hydrogen bonds with the surface of silica and therefore protect the cell membrane from damage (Nash *et al*, 1966). Allison (1971) proposed the theory of silica-lipid interaction as the mechanism of membrane damage. Weissmann and Rita (1972) lent support to this theory by observing that silica has the capacity to disrupt liposomes prepared from the lipids lecithin, cholesterol and dicetylphosphate. Gabor and Anca (1974) suggested peroxidation of membrane lipids as the mechanism by which silica causes damage; although Summerton *et al* (1977) found that vitamin E, a known inhibitor of lipid peroxidation, afforded no protection against membrane damage. Results from the report by Summerton *et al* (1977) suggested a silica-protein rather than a silica-lipid interaction as the mechanism of membrane lysis. They proposed that silica interacts with the erythrocyte membrane by repulsing sialic groups on the membrane, lysis occurring by removal of protein material. An analysis of adsorbed material surrounding the surface of the silica particles following erythrocyte lysis showed the presence of protein.

A study by Depasse (1978) suggested that the sensitivity of an erythrocyte to silica was dependent on its sialic acid content, and also on the electrostatic repulsion between negatively charged particles and the membrane. However, further work by Depasse (1977, 1980) also indicated a role for the trimethylammonium group ($-N(CH_3)_3^+$) of the membrane lecithin molecule; it was proposed that silica may interact with this group, thus causing membrane lysis. A more recent study by Nolan et al (1981) further confirms the contribution of the negative charge of the silica surface to the haemolytic capacity of the dust. However, it must be concluded that the mechanism of interaction of silica with the cell membrane is by no means proven, and requires further investigation.

1.13.4.2 The Haemolytic Activity of Asbestos.

The variation in the haemolytic ability of different types of asbestos, despite the fact that they all have the capacity to induce fibrosis in vivo, has initiated a considerable amount of research into the mechanism of interaction of these fibres with the cell membrane (Harington et al, 1975). Harington et al (1971b) examined this problem, and concluded that the haemolytic capacity of a fibrous dust was governed by its magnesium content; they proposed that the magnesium reacts with the sialic acid residues of the cell membrane. The finding that the addition of either EDTA or sialic acid to the culture system led to an inhibition of haemolysis (Harington et al, 1971b) lent support to this theory; a close correlation between the haemolytic capacity and the magnesium content of the fibre samples was also observed.

In 1975, Harington et al reviewed the theory of osmotic lysis as the mechanism of interaction of chrysotile with the cell membrane. Using the fluid mosaic model of membrane structure (Singer and Nicolson, 1972), it was suggested that the interaction between chrysotile and sialic acid would lead to a clustering of the membrane glycoproteins, thereby leading to an increased permeability of the membrane to sodium ions. Such an influx of ions into the erythrocyte would result in an increase in the intracellular osmolarity and eventually lead to rupture of the cell by osmotic lysis. Evidence to support this was later provided by Sykes et al

(1980) who showed that haemolysis by chrysotile was inhibited both by low temperatures (15°C) and by the addition of sucrose to the culture system.

A number of studies have been reported in which the effect of altering the surface properties of asbestos has been found to change the haemolytic capacity of the fibres. Desai et al (1975) and Jaurand et al (1979b) showed that the adsorption of erythrocyte phospholipid membranes, serum or pulmonary surfactant onto the surface of the chrysotile fibre effectively reduced the haemolytic capacity. Leaching chrysotile in acid reduced its haemolytic capacity, whilst leaching crocidolite or amosite increased their haemolytic abilities (Jaurand et al, 1979b). These results have been explained in terms of the surface charge, or zeta potential, by Light and Wei (1977a, 1977b, 1980). Light and Wei have shown that the haemolytic ability of the different types of fibre was closely related to the particular value of the zeta potential, i.e. chrysotile>amosite>crocidolite. In particular, whilst untreated chrysotile was more haemolytic than crocidolite, the leaching of the fibres resulted in a decrease of both the zeta potential and haemolytic ability of chrysotile, and an increase in both zeta potential and haemolytic ability of crocidolite. Light and Wei (1980) also showed that pulmonary surfactant was readily adsorbed onto the surface of chrysotile fibres and this comprised a decrease in both the zeta potential and the haemolytic ability of the fibres. It has been suggested by Jaurand et al (1979b) that the haemolysis caused by chrysotile asbestos is a self-limiting process, due to the ability of the fibre to interact with the membrane thereby leading to the extraction of lipids which then adsorb onto the surface of the fibre. A recent report by Depasse (1982) suggests that an interaction between chrysotile and the lipid-bound sialic acid portion of the membrane is responsible for the observed haemolysis.

As in the case of silica, there is much dispute concerning the mechanism by which asbestos interacts with the cell membrane. There is evidence to suggest that the fibre surface may cause membrane damage by interaction with membrane proteins and/or lipids (Jaurand et al, 1979b; Depasse, 1982). Further research may help to elucidate this problem.

1.13.5 The Macrophage.

The realisation that the alveolar macrophage is one of the first cells to ingest inhaled asbestos fibres in vivo, has stimulated an extensive examination of the direct effects of different types of asbestos fibres on this cell in vitro (reviewed in Harington et al, 1975; Harington, 1976; Miller, 1978). In particular the ability of asbestos to either kill, or alternatively modulate the release of various cellular secretions has been examined; attempts have been made to relate any observed effects to either the surface properties of alternatively the fibre dimensions of asbestos (Miller, 1978). Another dust that is well known for its fibrogenicity in vivo and cytotoxicity towards macrophages in vitro is silica (Harington et al, 1975), and a comprehensive examination of the in vitro interaction of silica particles with the macrophage has been undertaken. It has therefore proven relatively impossible to consider the effect of asbestos on this cell, without also paying attention to the action of silica, as establishing the mechanism of interaction of one type of dust may serve to elucidate the mode of action of others.

1.13.5.1 Silica and the Macrophage.

In 1957, Marks reported that silica had a toxic effect on macrophages in vitro, and the suggestion that the cytotoxicity displayed towards macrophages in vitro might reflect the fibrogenic potential in vivo (Marks and Nagelschmidt, 1959) ensured the continued use of this type of system as a predictive assay for fibrogenicity. A number of later reports, however, have displayed a greater concern for establishing the

exact mechanism by which silica induces cell death. Using phase-contrast microscopy and histochemical staining techniques, Allison et al (1966) demonstrated that macrophages readily ingested silica particles, these were transferred to phagosomes and subjected to attack by lysosomal enzymes. After 24 hours of exposure to silica most of the lysosomal enzymes had escaped from the lysosomes and were released into the culture medium, and the majority of the macrophages were dead. It was therefore concluded that silica may kill the macrophage by lysing the phagolysosomal membrane, this would allow a release of enzymes into the cytoplasm, resulting in intracellular destruction and autolysis (Allison et al, 1966).

The observation that silica may kill macrophages more rapidly in the absence of serum than in its presence gave rise to the idea of rapid and delayed cytotoxicity (reviewed in Harington et al, 1975). Allison (1971) demonstrated, by staining with fluorescent antibodies against serum proteins, that silica particles may become coated with serum proteins. Upon phagocytosis by macrophages the coating proteins were digested away from the particles by lysosomal enzymes, thereby resulting in the presentation of the silica surface to the lysosomal membrane. It was proposed by Allison (1971) that in the absence of serum, the surface of the uncoated particles are allowed ready access to the macrophage membrane, this results in a rapid disruption of the membrane and release of intracellular constituents. A later study by Davies (1980b) has confirmed that silica has the ability to induce enzyme loss from macrophages, and following phagocytosis, the release of lysosomal enzyme was paralleled by the release of the cytoplasmic enzyme lactate dehydrogenase (LDH).

A number of different methods have been used to study the viability of macrophages following dust ingestion, these have generally comprised failure to exclude vital dyes, inability to metabolize 2,3,5-triphenyltetrazolium chloride (TTC), decreased oxygen consumption or a failure to split fluorescein esters (Parazzi et al, 1968a,b; Allison, 1971; Robock and Klosterkötter, 1973), and they have all confirmed the cytotoxic nature of silica.

A recent report by Kane et al, (1980) has refuted the association between silica induced lysosomal rupture and cell death. Kane et al (1980) used the $P338D_1$ macrophage-like cell line, and showed that silica had the ability to rupture the lysosomal membrane, but cell death did not occur unless calcium ions were present in the culture medium; in addition, the use of indomethacin to stabilize the lysosomal membrane did not reduce silica-induced cytotoxicity. It was concluded that silica may alter the ability of the membrane to maintain a low permeability to calcium, and the resulting influx of ions would involve metabolic deterioration and cell death (Kane et al, 1980).

A number of mechanisms have been proposed by which silica may interact with and damage the macrophage membrane. The observation that the polymer PVPNO can effectively protect macrophages from the cytotoxic action of silica (Allison et al, 1966) prompted the theory that the particles may interact with the macrophage membrane via hydrogen bonding of silicic acid to lipid and protein components. The structure of silicic acid comprises a number of hydroxyl groups which could form hydrogen bonds with a number of membrane components, for example the phosphate esters on phospholipids; this type of bonding would cause considerable disruption of any exposed cell membrane. Nash et al (1966) demonstrated that PVPNO is an efficient electron acceptor and may therefore form hydrogen bonds with silicic acid; such preferential bonding would reduce the possibility of silicic acid interacting with the membrane and thus a protective effect would occur.

Munder et al (1966, 1967) examined the effect of silica on phospholipid and lipid metabolism. Phagocytosis of quartz resulted in an accumulation of surface-active lysophosphatides, and Munder et al (1966, 1967) suggested that quartz may potentiate activation of the phospholipase A system. Certainly quartz could have the ability to activate membrane lipid or enzymes by electron transfer (Robock, 1968). Munder and Lebert (1977) later reported that silica could not only inhibit membrane-bound acyl-transferase, but also activate the phospholipase A system. It can therefore be

postulated that silica stimulates the release of phospholipase A, this results in a breakdown of lecithin and cephalin triglycerides to produce an accumulation of membrane-active fatty acids and lysophosphatide. Silica also inhibits acyl-transferase, thus the lipids cannot be inactivated by this enzyme and membrane disruption occurs (Munder and Lebert, 1977).

Gabor et al (1980) examined further the involvement of lipid peroxidation in silica induced toxicity by demonstrating that vitamin E could effectively protect the macrophage from the cytotoxic action of quartz. The phytyl chains in α-tocopherol interact with membrane phospholipid, and this results in a membrane stabilization. The treatment of silica-exposed rats with vitamin E involved a reduction of both lipid peroxide and hydroxyproline levels in the lung (Gabor et al, 1980). Presumably the reduced lipid peroxide levels indicated the effectiveness of vitamin E at reducing the macrophage rupture by silica. In addition, the abrogation of macrophage death resulted in a reduction in fibrosis, as manifest by the lowered hydroxyproline (ie. collagen) levels.

Despite numerous theories, investigations into the cytotoxic action of quartz are still in progress. A number of mechanisms have been proposed by which quartz may interact with the macrophage, all are supported by experimental evidence, but none as yet, are completely proven. It must be borne in mind, however, that similar types of mechanism may be brought into play during the interaction of asbestos fibres with the macrophage membrane; current theories concerning the in vitro effects of asbestos fibres on macrophages will be described in the following sections.

1.13.5.2 The Cytotoxic Effect of Different Asbestos Types Towards Macrophages and the Induction of Selective Release

The first examination of the ingestion of asbestos by phagocytic cells occurred in 1883 when Metchnikoff fed asbestos fibres to amoeboid sponge cells in vitro; unfortunately he failed to record either the type of asbestos used or the cytotoxic nature of the fibres.

It was not until fifty years later that research workers started to seriously examine the modulating effect of different types of asbestos on various defined indicators of the metabolic functioning of macrophages. Koshi et al (1968, 1969) exposed macrophages to various forms of asbestos for up to 3 hours in the absence of serum. They reported that chrysotile induced an increased release of acid phosphatase and a decrease in lactate production, whereas the amphiboles did not; and it was therefore concluded that the amphiboles were less cytotoxic than serpentine. Beck et al (1971) examined the effect of chrysotile on guinea pig peritoneal and alveolar macrophages; the fibres induced an increase in eosin uptake, lactate dehydrogenase (LDH) release and lactate production, thereby suggesting that the permeability of the membrane had increased. Bey and Harington (1971) treated eight day old cultures of hamster and peritoneal macrophages for 72 hours with UICC crocidolite, amosite and chrysotile. A morphological examination of the cells suggested that the amphiboles had not exerted any cytotoxicity, whereas chrysotile caused an increased vacuolation of the cells, a loss of ruffles on the cell membrane, and some macrophages were pyknotic in appearance.

Allison (1971) examined the effects of UICC samples of asbestos on cultures of mouse peritoneal macrophages. He found that chrysotile had the ability to kill cells within 2 hours in the absence of serum, compared to 48 hours for the dust to take effect in the presence of 10% serum. Allison postulated that the "early cytotoxicity" could be due to a direct interaction of the fibres with the plasma membrane; whereas, in the presence of serum, protein coating the surface of the chrysotile fibres would protect the cell membrane until the fibres entered the lysosomes, and a "late cytotoxicity" would therefore be seen. Robock and Klosterkötter (1971, 1973) and Klosterkötter and Robock (1975) also examined the effect of UICC samples of asbestos on guinea pig peritoneal macrophages. Using tetrazolium reduction as their estimate of cell damage, they showed that chrysotile (A and B) was more cytotoxic than crocidolite, amosite

or anthophyllite, and anthophyllite was the most cytotoxic of the amphiboles examined. They confirmed the early cytotoxic effect of chrysotile in absence of serum, but did not succeed in abrogating the effect by adding fresh serum. Reviews by Harington et al (1975) and Miller (1978) have suggested a reason for this finding; Robock and Klosterkötter did not heat-inactivate their serum, and as asbestos has the ability to activate serum complement components (Harington et al, 1971a), the generation of cytotoxic complement components may have occurred in Robock and Klosterkötter's system which would effectively reduce the viability of the cells.

A morphological study of the ingestion of chrysotile by macrophages showed that secondary lysosomes formed following the phagocytosis of fibres, and these lysosomes apparently fused with the cell membrane and released their contents into the culture medium (Allison, 1973). Davies et al (1974 a) reported that chrysotile had the ability to induce a release of lysosomal enzymes into the culture fluid, but, more interestingly, this was in the apparent absence of any loss of the cytoplasmic enzyme LDH. This type of release was termed a "selective release" of lysosomal enzymes, and was destined to be used in connection with the in vitro effects of chrysotile for a number of years. A number of earlier reports had already examined the induction of lysosomal enzyme release by chrysotile, although this release was not compared with cytoplasmic enzyme levels. Miller and Harington (1972) measured the levels of acid phosphatase in stimulated hamster peritoneal macrophages after 16 hours of exposure to asbestos; chrysotile induced a release of enzyme into the medium, whereas crocidolite, amosite and the rutile control did not. Kaw and Zaidi (1975) found that anthophyllite, tremolite and actinolite were relatively inactive towards alveolar and peritoneal macrophages, whereas chrysotile caused an increased acid phosphatase release accompanied by a corresponding uptake of vital dye.

The report that chrysotile may initiate a selective release of lysosomal enzymes from macrophages (Davies et al, 1974 a) stimulated

considerable interest, and has resulted in an extensive examination of this phenomenon by a number of research groups. Morgan et al (1977) measured glucosaminidase, galactosidase, glucuronidase and LDH levels in mouse peritoneal macrophages, 24 hours following exposure to chrysotile, and confirmed the findings of Davies et al (1974 a). A conflicting result was reported for the alveolar macrophage however. Jaurand et al (1978) and Kang et al (1979) reported a loss of cytoplasmic LDH and phosphohexose isomerase from macrophages following chrysotile ingestion, thus suggesting a loss in viability. A later study by Jaurand et al (1980 a) showed that a low dose of chrysotile (50μg/ml) may induce a selective release of β-galactosidase, whereas LDH was released at higher concentrations of dust only. Morgan and Allison (1980) postulated that the differences in result may be due to the use of macrophages from different sources. They compared the response to asbestos of alveolar and peritoneal macrophages from different animals and found a generally similar response, in that lysosomal enzyme release proved to be both dose and time dependent. It was suggested, however, that some differences should be expected due to the use of cells from a variety of different sources and also dissimilar culture techniques.

In addition to the release of hydrolytic enzymes it has been shown that asbestos can stimulate the production of a number of other substances by macrophages. Miller and Harington (1972) reported that chrysotile ingestion by peritoneal macrophages resulted in an increase in the lysolecithin levels, and this was accompanied by a corresponding decrease in lecithin and phosphatidylethanolamine. It was postulated that this effect may be due to the release of phospholytic and lysolytic enzymes; a later study by Sirois et al (1980) confirmed that chrysotile may stimulate the release of phospholipase A, and this could contribute considerably to the production of surface-active lysophosphatides. It has also been shown that chrysotile can stimulate the release of prostaglandin E_2 (Sirois et al, 1980) and plasminogen activator (Hamilton, 1980);

both of these substances play an important role in inflammatory processes (Taussig, 1979).

The consistent finding that chrysotile can stimulate the release of various enzymes from macrophages either via a cytotoxic interaction or by selective release, has led a number of people to try and discover the mechanism of action. In order to try and explain why chrysotile has such a different effect to the amphiboles, the surface properties of these asbestos types have been examined in relation to their respective cytotoxic potentials.

1.13.5.3 The Role of the Surface Properties of Asbestos in the Cytotoxic Activity Towards Macrophages.

A number of studies have been concerned with an examination of the mechanism of action of asbestos; these have often comprised an alteration of the surface properties of the fibres in order to see if the *in vitro* effects become modified. An alteration of the surface properties may be attained by coating the fibres with a number of agents, or denaturing the surface by the use of either high temperatures or strong acids.

Miller and Harington (1972) postulated that the magnesium ions on the surface of the chrysotile fibre may interact with negatively charged glycolipids on the plasma membrane, thereby initiating a cytotoxic reaction. The suggestion was supported by Kaw and Zaidi (1975) who reported that the acid polymer carboxymethyl cellulose (CMC) could effectively reduce the cytotoxic effect of chrysotile. This result was attributed to the ability of CMC to chelate the magnesium ions from the surface of the fibres (Kaw and Zaidi, 1975). The use of PVPNO, an agent which can protect macrophages against the action of silica (Allison *et al*, 1966), was completely ineffective, however, at reducing the effect of chrysotile (Schlipköter, 1968; Beck *et al* 1971 ; Robock and Klosterkötter, 1971, 1973). Davies (1980b) examined the response of macrophages to treatment with fibres of crocidolite coated with the lung surfactant component dipalmitoyl-α-lecithin (DPL). The release of the lysosomal

enzyme ß-glucuronidase was significantly greater than the normal value seen for uncoated crocidolite. Whilst this finding has not elucidated the mechanism of action of the fibre, it has demonstrated that the pattern of enzyme release observed *in vitro* may not necessarily reflect that seen *in vivo*. Inhaled fibres may become coated with surfactant in the lung prior to ingestion by alveolar macrophages, and thus a different response might be seen.

Altering the surface properties of chrysotile by either heating or acid treatment had a dramatic effect upon its toxicity. Robock and Klosterkötter (1971, 1973) reported that chrysotile A that had been heated to 600°C showed an increased degree of early cytotoxicity towards macrophages. Hayashi (1974) confirmed the increased toxicity of chrysotile upon heating to between 650 and 800°C, and it was suggested that this may be related to the disordered state of the chrysotile that occurs in the process of transformation to forsterite during heating. Morgan *et al* (1977) examined the effect of leaching chrysotile in acid on the ability of the fibres to induce selective release. They showed that removal of up to 55% of the magnesium by leaching caused an increase in selective release, but upon removal of more than 55% of magnesium the enzyme release declined. Beck *et al* (1971) and Jaurand *et al* (1980a) reported that leaching chrysotile in acid increased its cytotoxic potential, and it was proposed that the removal of magnesium resulted in the exposure of the Si-OH layer (Jaurand *et al*, 1980a). Certainly, the evidence from macrophage studies would suggest a role for the surface properties of a fibre in determining its cytotoxic potential, however the mechanism of action has not been clarified. A number of research groups have used the erythrocyte membrane as a model for examination of fibre/membrane interactions (Section 1.13.4). There would appear to be a role for the magnesium content of the fibres (Harington *et al*, 1971b) as well as the surface charge (Light and Wei, 1980) in determining the cytotoxic potential, and this may be due to interactions with the lipid-bound sialic acid portion of the membrane (Depasse, 1982).

However, further work is still required to establish if the results obtained from haemolysis experiments can reflect the interaction of fibres with macrophages.

An anomolous result has become apparent upon comparison of *in vitro* data with *in vivo* data. Chrysotile has proven considerably more cytotoxic *in vitro* than the amphiboles, but all of the asbestos types have the capacity to initiate fibrosis and cancer *in vivo* (Harington *et al*, 1975). There is epidemiological (Section 1.11.2) and experimental (Section 1.12.2) evidence available to suggest that the fibre dimension may also play a part in determining the pathogenicity, and a number of research groups have been involved in relating the length of a fibre to its cytotoxic activity towards macrophages.

1.13.5.4 Asbestos Cytotoxicity Towards Macrophages and its Relationship with Fibre Dimensions

In recent years there has been a definite trend towards examining the relationship between the fibre length of an asbestos sample and its cytotoxic potential. Davies (1980a) and Davies *et al* (1980) studied the response of mouse peritoneal macrophages to treatment with samples of UICC crocidolite, amosite and anthophyllite that had been ball-milled for different periods of time. They showed that the release of β-glucuronidase and LDH progressively decreased with increased time of ball-milling for each sample. Ball milling is known to reduce the overall fibre length of a fibrous sample (Brown *et al*, 1978), and this result would suggest that longer fibres stimulate enzyme release. Davies (1980b) also examined the effect of three samples of tremolite on enzyme release, and found that the sample which contained a higher proportion of longer fibres caused a significantly greater release of lysosomal enzyme than of cytoplasmic LDH. The two tremolite samples which contained relatively short fibres did not show this type of release. Chamberlain *et al* (1979) examined the effect of a number of fibrous Dawsonite (dihydroxyaluminium carbonate) samples on

macrophages, and found a close correlation between enzyme release and the number of fibres greater than 10 μm in length and less than 1.4 μm diameter in each sample.

Beck and Tilkes (1980) prepared three sub-fractions for each of the UICC crocidolite, amosite and chrysotile types, and each sub-fraction contained different quantities of long fibres. When phagocytic, ascitic tumour cells were treated with these samples, an increased toxicity was observed as the proportion of longer fibres in the samples increased (Beck and Tilkes, 1980). When these studies were extended to macrophages it was shown that the longer fibres were more cytotoxic than the shorter ones (Kaw et al, 1982), although a threshold length for cytoxicity was not suggested.

Johnson and Davies (1980 , 1981) used transmission electron microscopy techniques to examine the phagocytosis of fibres by macrophages. They reported that ingested mineral fibres could be found protruding from membrane-bound vacuoles, lying free in the cytoplasm, and also penetrating the nucleus. Johnson and Davies (1980, 1981) suggested that the rigid nature of the fibre may cause considerable internal damage during cell movement, and that the partially engulfed longer fibres (>8μm) would, in particular, prove the most harmful.

The current evidence would suggest that the dimensions of a fibrous sample play an important part in determining the cytotoxicity towards macrophages. There is however a dearth of data to substantiate the proposal that a threshold length exists, above which the fibre may become cytotoxic towards macrophages. To try and clarify this situation, a number of research groups have employed permanent cell lines, not only for the purpose of determining the relationship between fibre length and cytotoxicity, but also to establish a reliable assay for the prediction of the fibrogenicity and carcinogenicity of dust sample.

1.13.6 The Use of Permanent Cell Lines, and Their Role in the Determination of Pathogenic Fibre Lengths

One of the first series of studies designed to investigate the use of a permanent cell line as both a macrophage substitute and a possible predictive assay system was reported by Wade et al (1976, 1980). They used the phagocytic macrophage-like cell line, the $P388D_I$ cell which was derived from a mouse lymphoma (Dawe and Potter, 1957); and their assessment of degree of cytotoxicity involved a direct count of alteration in cell numbers at the end of 72 hours exposure to fibres. The amphiboles proved less cytotoxic than chrysotile, and this effect was attributed to a greater number of fibres being available in the chrysotile sample (Wade et al, 1976). An examination of the effect of these three types of asbestos on the integrity of the $P388D_I$ cell membrane by direct measurement of the cell membrane electrophysiology, confirmed that chrysotile had the ability to reduce both cell membrane potential and cell input resistance more rapidly than amosite or crocidolite (Gormley and Wright, 1980); this would agree with the finding of Wade et al (1976). The cytotoxic response of $P388D_I$ cells to samples of Dawsonite fibres of varying aspect ratios was also examined (Wade et al, 1980). The order of cytotoxicity paralleled the order of probability that the samples could induce pleural sarcomas in rats. The results tended to agree with the Stanton Hypothesis (Stanton et al, 1977; Stanton and Layard, 1978) that those fibres >8μm in length and less than 0.25μm diameter would be the most pathogenic (Wade et al, 1980; Lipkin, 1980). A comparison between the response of $P388D_I$ cells, alveolar and peritoneal macrophages to treatment with dust, suggested, on the basis of enzyme release, that the order of sensitivity for these three cell types was $P388D_I$>alveolar>peritoneal macrophages (Daniel and Le Bouffant, 1980). The primary macrophage cultures showed some variation in result due to unspecified "uncontrolled factors", and it was concluded that the high reproducibility and sensitivity of the $P388D_I$ cell line would make it particularly suitable for use in a cytotoxicity assay system.

Chamberlain and Brown (1978) examined the response of two non-phagocytic cell lines to treatment with mineral fibres, these being the V79-4 Chinese hamster lung cell, and A549 human alveolar Type II lung cell. In these systems a carcinogenic dust was defined as having the ability to reduce colony formation by V79-4 cells, and induce the formation of giant cells by A549 cells (Brown et al, 1979a, b; Chamberlain et al, 1979). Using this system it has been shown that fibre size is important in determining the cytotoxic potential of crocidolite, amosite, anthophyllite (Brown et al, 1978), glass fibres (Brown et al, 1979 a) and Dawsonite fibres (Chamberlain et al, 1979). A fibre length threshold for in vitro pathogenicity was proposed, the cell response to Dawsonite fibres correlated well with fibre dimensions of >10μm length and <1.4μm diameter, and for asbestos samples those fibres of >6.5um length proved the most pathogenic. A close association between in vitro cytotoxicity and in vivo carcinogenicity for a number of fibrous samples was observed (Chamberlain and Brown, 1978; Brown et al, 1979b; Chamberlain et al, 1980), thus indicating that both cell types may prove useful in a predictive assay system. Neither the V79-4 or the A549 system has shown cytotoxic susceptibility to fibrogenic particulates such as silica (Chamberlain and Brown, 1978; Davies et al, 1980); it could therefore be postulated that both cell types may be used in vitro to differentiate between those dusts that are fibrogenic but not carcinogenic in vivo (Chamberlain et al, 1980; Davies et al, 1980).

The use of permanent cell lines in the in vitro studies of asbestos/cell interactions has a number of advantages that would recommend their use; these being a consistent reproducibility upon the establishment of the appropriate assay system (Wade et al, 1976; Brown and Chamberlain, 1980), and the removal of any inherent variability that may be seen during the use of primary cells (Daniel and Le Bouffant, 1980).

There is evidence to suggest that there might be a link between the dimensions of a fibrous sample, its *in vitro* cytotoxicity and also its *in vivo* pathogenicity (Wade *et al*, 1980; Chamberlain *et al*, 1980). This requires further comparison of the *in vivo* and *in vitro* pathogenicity of samples of fibrogenic, carcinogenic and non-pathogenic dusts of known dimensions, before the use of any of these systems as predictive assays will be fully validated.

One of the primary cells that has been extensively examined with respect to its response to treatment with asbestos fibres, is the macrophage (Section 1.13.5). In general this cell has been examined with respect to its ability to phagocytose asbestos fibres and release intracellular enzymes. The macrophage, however, performs a large number of functions in the body, and whilst only a few of these will be examined in the main body of this thesis, it is important to bear in mind that other functions are known. The role of the macrophage will be discussed in the following section.

1.14 The Role of the Macrophage in the Body.

For a number of years the important role of the macrophage in defence of the body against invading pathogens and disease has been recognised (see review by Nathan *et al*, 1980). This bodyguard is professionally geared towards the recognition, ingestion, destruction and removal of inhaled organisms or particles. As well as endeavouring to protect the body against proliferating tumour cells and disease, the phagocyte is also involved in extensive communication with other cellular and also humoral members of the immunological defence league of the host (these aspects are reviewed in Van Furth, 1980a). The alveolar macrophage is involved in both the ingestion of inhaled asbestos particles and their removal from the lung, because of this it is important that the effects of asbestos fibres on this particular cell type as well as their protective role in the body be considered.

1.14.1 The Differentiation, Activation and Heterogeneity of the Macrophage.

It has been established that the macrophage can be found in a variety of tissues in the body, including the connective tissue, lung, liver, lymph nodes, spleen, bone marrow, skin and other organs (Van Furth, 1980b). Recent reports (for a review see Van Furth, 1981) suggest that the majority of macrophages originate in the bone marrow, where they develop from committed stem cells into monoblasts. The monoblasts differentiate to form monocytes which pass into the blood stream and after a period of time migrate into the tissues where they develop further to become mature macrophages. During an inflammatory response at a given site in the body, the number of mononuclear phagocytes increases; although the degree and kinetics of the rate of increase may vary according to the nature of the stimulus (Van Furth, 1980b). When monocytes have reached an inflammatory site they may develop further to become resident macrophages, transform into epithelioid cells, or fuse with other mononuclear phagocytes to become multinucleate giant cells (Papadimitriou and Walters, 1979).

Recently it has become evident that macrophage populations, upon maturation, may develop different functional properties, depending upon their situation in the body and on the nature of any localised stimulatory agents (Hopper et al, 1979). An unstimulated or resident macrophage may become "activated" upon contact with a variety of agents such as endotoxin, antigen, lymphokines, bacteria, protozoa and other agents (Nelson et al, 1978). A number of properties of activated macrophages differentiate them from resident or unstimulated cells, and these are listed in Table 1.7. In general it can be said that activated macrophages exhibit an increased metabolic activity, enzyme content, secretory ability and microbicidal and tumouricidal activity.

Table 1.7 Properties of Activated Macrophages.

Morphological increases in:

- adhesiveness and spreading
- ruffled membrane activity
- cytoplasmic granules

Biochemical increases in:

- adenyl cyclase
- cyclic GMP
- Ca^{++} influx
- glucose oxidation
- glucose uptake
- lysosomal enzymes
- lysosomal enzyme release
- lactic dehydrogenase
- lysozyme
- collagenase
- elastase
- plasminogen activator production
- prostaglandin production

Functional increases in:

- pincytosis
- phagocytosis
- intracellular microbicidal activity
- cytotoxic effects on tumour cells

Adapted from Hopper et al (1979)

A number of differences have been shown to exist between populations of macrophages isolated from different areas of the body. In particular, alveolar and peritoneal macrophages have been compared, and it is evident that whilst the metabolism of the alveolar macrophage is dependent upon aerobic oxidative phosphorylation, peritoneal cells utilize the glycolytic pathway to obtain their energy requirements (Karnovsky et al, 1970) Alveolar macrophages also have higher levels of hydrolytic enzymes than peritoneal macrophages (Leake et al, 1964), a higher elastase production by resident cells (White et al, 1977), a lower number of Fc receptors (Rhodes, 1975), and a more efficient accessory cell activity (Gorenberg and Daniel, 1978).

As well as the obvious heterogeneity noted between different macrophage populations, heterogeneity has also been reported within individual populations (Förster and Landy, 1981). Lee and Berry (1977) separated Corynebacterium parvum activated mouse peritoneal macrophages by a sedimentation velocity technique and demonstrated that the larger cells were more cytostatic towards tumour cells than the smaller cells; this observation was later confirmed by Miller et al (1980) and Morahan and Miller (1981).

It is therefore evident, when considering the activity of a population of macrophages during phagocytosis, digestion, secretion or immunological mediation, that the response of each individual cell within the population might differ, as well as the response between different populations, depending on the origin of the given population and also on the nature of prior stimulating agents.

1.14.2 Phagocytosis by Macrophages

An important activity of the macrophage is its ability to recognise and phagocytose particulate matter. Macrophages are known to possess surface receptors which recognise the Fc portion of the IgG immunoglobulin molecule, a fragment of the C3 molecule

(Reynolds et al, 1975), and non-specific receptors which mediate the attachment of particles such as latex beads (Weir and Ogmundsdottir, 1977; Benoliel et al, 1980). A number of reports have shown that these receptors not only allow attachment of the appropriately coated (opsonized) particle to the macrophage membrane, but also mediate its internalization (Mantovani et al, 1972 ; Huber et al, 1968; Murphy et al, 1979). Bianco et al (1975) reported that C3b allows the binding of particles to the surface of both resident and activated macrophages, but initiates phagocytosis in activated macrophages only. The Fc receptor mediates phagocytosis in both resident and activated cells (Bianco et al, 1975), although the number of Fc receptors is increased upon activation of the cell (Rhodes, 1975).

Ingestion appears to be a microfilament-mediated process (Michl and Silverstein, 1978) initiated by attachment of the particle to the appropriate surface receptor and involving successive attachment of receptors to ligands on the circumference of the particle. This has been described as the "zipper mechanism" of phagocytosis by Griffin et al (1976). The process of phagocytosis involves some considerable internalization of the plasma membrane which is replaced after a period of several hours (Werb and Cohn, 1972), and culminates in the encasement of the phagocytosed particle within a phagolysososomal vacuole.

The response of a cell, following phagocytosis of a particle may vary depending upon the nature of the particle involved, and some of these responses will be discussed in the following sections.

1.14.3 The Macrophage and its Secretory Products

The macrophage is well-known for the large variety of secretory products released either in a constitutive manner or in response to various stimuli (Table 1.8). The secretory ability of the macrophage enables it to take part in a number of interactions which involve both cellular and humoral components of the inflammatory and immune responses (Nathan *et al*, 1980). The release of a small number of these secretory products will be discussed in this section.

Heise and Myrvik (1967) established that macrophages secrete a bactericidal agent called lysozyme, and the enzyme may be released at a constant rate *in vitro*, and is not related to a phagocytic stimulus (Gordon *et al*, 1974). A number of acid hydrolases can also be released in a constitutive manner (Schnyder and Baggiolini, 1978); although it has been shown that lysosomal hydrolase secretion may be significantly increased upon attachment and/or phagocytosis of various particles (Davies and Bonney, 1980). The phagocytosis if inert particles such as latex and sheep erythrocytes did not appear to initiate a selective release of lysosomal enzymes (Axline and Cohn, 1970), although Morland (1979) noted an increased loss of Cathepsin D upon phagocytosis of latex. A number of inflammatory agents such as zymosan (Weissman *et al*, 1971), dental plaque (Page *et al*, 1973), *Micropolyspora faeni* (Schorlemmer *et al*, 1977c), chrysotile asbestos (Davies *et al*, 1974a) and antigen-antibody complexes (Cardella *et al*, 1974) can initiate the selective release of such enzymes as acid phosphatase, β-glucuronidase and β-glucosaminidase in the absence of any loss of cellular lactate dehydrogenase (LDH). Schorlemmer *et al* (1976) and Schorlemmer and Allison (1976) reported that the complement component C3b can cause selective release of lysosomal enzymes; in addition, the ability of various forms of dextran sulphate to induce selective release appeared to vary according to the ability of each form to activate complement (Schorlemmer *et al*, 1977 b) , and the hypothesis was advanced that selective release may be initiated upon activation of the alternative pathway by the appropriate agent. More recently however, Riches and Stanworth (1982)

Table 1.8 Some Secretory Products of Mononuclear Phagocytes

Enzymes:

- lysozyme
- neutral proteases
- acid hydrolases
- arginase

Complement components
Enzyme inhibitors
Binding proteins
Nucleosides and metabolites
Reactive metabolites of oxygen
Bioactive lipids
Factor chemotactic for neutrophils
Factors regulating the synthesis of proteins by other cells
Factors promoting the replication of lyphocytes and fibroblasts
Factors inhibiting the replication of lymphocytes and tumour cells

Adapted from Nathan *et al* (1980)

have shown that selective release can be initiated by the compounds chloroquine and benzimidazole which do not have the ability to activate complement. It was proposed that secretion may be initiated by alteration of the pH within the lysosome (Riches and Stanworth, 1982). Bonney et al (1978) have also shown that the degree of selective release may alter depending on the nature of any prior stimulation of the macrophage before phagocytosis occurs.

The release of a number of neutral proteases by macrophages has been reported, including elastase (Werb and Gordon, 1975), collagenase (Wahl et al, 1974), angiotensin converting enzyme (Silverstein et al, 1978) and plasminogen activator (Gordon et al, 1974). The phagocytosis of indigestible substances such as chrysotile can modulate the secretion of plasminogen activator (Hamilton, 1980), and macrophages exposed to a phagocytic stimulus will sometimes secrete plasminogen activator after a latent period of several days (Schnyder and Baggiolini, 1978).

Davies and Bonney (1980) have extensively reviewed the secretory properties of macrophages, and conclude that the rate of release of both neutral proteases and acid hydrolases can vary depending upon the nature of any prior stimulation of the macrophage populations, and can also be modulated by the presence of a non-digestible particle in the phagolysosome. This is of particular importance when considering the effect of ingestion of asbestos fibres upon cell secretion.

1.14.4 The Antimicrobial and Antitumour Activity of the Macrophage

Macrophages are actively involved in the destruction of both microbes and tumour cells in vivo (Nathan et al, 1980). It has been suggested that the definition of an activated macrophage might be given in terms of its enhanced ability to destroy tumour cells and intracellular microbial pathogens (Mackaness, 1970; Nathan et al, 1979). The capacity of macrophages to kill intracellular microbes has, in a number of cases, been attributed to its ability to produce a variety of oxygen derivatives such as H_2O_2 and O_2^- (Murray et al, 1980), and a role for these intermediates in the destruction

of a number of intracellular pathogens such as Toxoplasma (Murray et al, 1980) Candida (Sasada et al, 1980) Leishmania (Murray, 1981) and trypanosomes (Tanaka et al, 1981) has been reported.

A variety of mechanisms have also been suggested by which the macrophage may destroy tumour cells; these include arginase secretion (Currie and Basham, 1978), the release of a proteolytic factor (Adams, 1980) and reactive oxygen metabolites (Nathan et al, 1979). As with antimicrobial activity, it appears that the antitumour activity of a macrophage is very dependent upon its activational state (Hopper et al, 1979), although the antitumour and antimicrobial activities of a macrophage do not necessarily parallel each other.

Resident macrophages are not generally found in an activated state, it is therefore necessary that these cells become triggered into a state of activation upon contact with any invading pathogen or antigen, before they attain the ability to destroy this material.

1.14.5 The Macrophage and the Immune Response

The regulation of the immune response following exposure to antigen is complex, involving considerable interaction between T and B lymphocytes and macrophages (Unanue, 1980). The process of phagocytosis of the antigen by the macrophage in general results in a re-expression of the antigen on its cell membrane, and this is recognised by T-cells to a much greater extent than if the antigen was available in a soluble form (Mitchison, 1969). For the T-cell to recognise the antigen, it must also be seen in association with macrophage membrane Ia antigen; this particular surface protein being genetically coded for by the I-region of the major histocompatability or transplantation locus of the species (Shevach, 1976). After the T-cell macrophage interaction, B-cells may become stimulated to produce antibody (Unanue, 1978), and other sub-sets of T-cells are stimulated to produce lymphocyte mediators or lymphokines which will prime or trigger the macrophages to achieve an adequate activational state for tumour cell killing or microbicidal destruction (Meltzer et al, 1982). Lymphokines will also attract

macrophages to the areas of macrophage/T-cell interaction (Unanue, 1980). The macrophage may remain in an activated state for a period of time after antigen invasion has subsided (Wing and Remington, 1978), and this may vary depending on the type of macrophage involved.

From the preceding brief review it can be seen that the macrophage plays an important role in the body. Not only is this cell involved in the phagocytosis and removal of inhaled particles from the lung, but also the destruction of invading microbes and tumour cells, the release of various enzymes and mediators which may modulate the activity of other cells in the body; and the macrophage also plays a central role in the immune system. It would therefore seem that the macrophage field is open for future research particularly with regard to the modulating effect that exposure of this cell to an environmental hazard such as asbestos might have on one or several of its functions. It is also evident that macrophage populations are heterogeneous in nature, and that the macrophage may be obtained in a variety of activational states; the response of the macrophage to treatment with asbestos may therefore vary, and this variation may be due to the source or the activational state of the cell.

1.15 Aims and Objectives

Asbestos is a fibrous silicate which can occur in a number of forms (Michaels and Chissick, 1979), and is of considerable commercial importance. The use of this mineral during the mining, milling or manufacturing procedures results in the generation of airborne fibres which may be inhaled by an exposed individual (Selikoff and Lee, 1978). Both epidemiological and animal studies have shown that all types of asbestos, regardless of their chemical structure, have the ability to initiate fibrosis and/or cancer (Becklake, 1976; Harington *et al*, 1975). In an attempt to clarify the mechanism of action of asbestos a number of workers have turned to the examination of the direct effects of fibres on potential target

cells in the body (Harington et al, 1975).

The macrophage is actively involved in the phagocytosis of asbestos fibres from the lung, and thus an extensive examination of the in vitro cytotoxic effects of asbestos on macrophages from a variety of sources has occurred (Harington et al, 1975). The cytotoxicity of asbestos fibres has generally been assessed in terms of release of intracellular enzymes into the culture fluid, and a link between enzyme release from macrophages in vitro and the ability of the fibres to initiate fibrosis in vivo has been suggested (Allison, 1968; Davies and Allison, 1976). Chrysotile has generally proven more cytotoxic than the amphiboles and this result has often been explained in terms of the more biologically active surface properties of the chrysotile fibres (Miller and Harington, 1972). Recent studies have, however, indicated that there is a role for the dimensions of a fibrous sample in determining its cytotoxic potential towards macrophages (Chamberlain et al, 1979; Kaw et al, 1982), V79-4 and A549 cells (Brown et al, 1978) and this is supported by both epidemiological (Timbrell et al, 1971) and animal studies (Klosterkötter, 1968; Wright and Kuschner,1977; Stanton and Layard, 1978). A number of fibre length thresholds have been suggested, above which the fibres may become pathogenic, and these are 8μm (Stanton and Layard, 1978), 10μm (Chamberlain et al, 1979), 6.5μm (Brown et al, 1978). However further work is required involving an examination of the effect of samples of defined fibre dimensions on macrophages; this type of study may provide further information concerning the fibre length threshold which may govern the cytotoxic potential of a fibre towards macrophages. This type of study is hampered to some extent by the observation that macrophages may be obtained in a variety of activational states, and this may modify the response of a cell to asbestos (Miller, 1978; Morgan and Allison, 1980). It is important therefore, to choose a population of macrophages of known activational state, or alternatively to use a permanent macrophage-like cell line (the reproducibility of which can assured) for use in a routine cytotoxicity study. It is also equally important to determine any differences in response which may occur by virtue of the activational state of the macrophage.

The main aims of this thesis are therefore:

1) To establish an accurate and reproducible method for the assessment of the fibre number and dimensions of asbestos samples.

2) To examine the use of the erythrocyte for determination of the mechanism by which fibre/membrane interaction occurs.

3) The establishment of a reproducible assay system using a macrophage population of known activational state, or a macrophage-like cell line, for routine examination of the cytotoxicity of asbestos samples samples. In this study cytotoxicity is defined as the occurrence of membrane damage and enzyme release. The fibre length threshold at which the fibres prove pathogenic towards macrophages will be assessed, as well as the usefulness of this system as a predictive assay.

4) The employment of electrophysiological techniques to examine macrophage/fibre interactions appears promising (Wright and Gormley, 1980) and this technique will be examined in order to determine if the inclusion of this technique in a permanent assay system would prove advantageous.

5) The assessment of any differences in response to asbestos that might be due to the activational state of the macrophage.

It is hoped that the following study will advance our knowledge with respect to macrophage activation and the response of the cell to asbestos; establish the relationship between fibre length and cytotoxicity towards macrophages, and indicate the usefulness of the in vitro system as a predictive assay for carcinogenicity and fibrogenicity.

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CHAPTER 2 THE ASSESSMENT OF THE DIMENSIONS OF ASBESTOS SAMPLES

2.1 INTRODUCTION.

There is a substantial quantity of epidemiological data available to demonstrate that all of the various types of asbestos are capable of initiating a variety of disease states in man (reviewed by Harington et al, 1975; Becklake 1976). Extensive in vivo studies (reviewed by Harington et al, 1975; Selikoff and Lee, 1978) have also provided evidence to show that all types of asbestos have the capacity to induce both fibrosis and cancer. The various asbestos types may be identified according to their very different physicochemical properties (Michaels and Chissick, 1979), and attempts to relate the pathogenicity of the asbestos types to these different properties have shown that the dimensions of the fibres may be involved. Epidemiological (Section 1.11.2) and experimental (1.12.2) studies have shown a possible role for the fibre length in determining the capacity of an asbestos sample to initiate disease in vivo, and in vitro studies have shown a role for the fibre length in inducing cellular cytotoxicity (Section 1.13.5.4 and 1.13.6). A number of research groups have demonstrated that the longer fibres in an asbestos sample may be cytotoxic towards both macrophages and P338D_1 cells (Davies 1979, 1980; Davies et al, 1980; Kaw et al 1982; Wade et al, 1980); and a close correlation between degree of enzyme loss and the number of fibres greater than 10μm has been shown (Chamberlain et al 1979). There is however, insufficient data available in the literature to support the hypothesis that a critical length exists at which fibres may become cytotoxic towards macrophages, and one of the aims of this thesis is to establish a relationship between in vitro cytotoxicity and fibre length. Before a study of this nature can be carried out, it is a prerequisite that a reliable and accurate assay system be established for the assessment of the dimensions of fibrous samples. There are a number of methods by which fibre dimensions may be examined, and these comprise the utilisation of either light, scanning (SEM) or transmission (TEM) electron microscope techniques. The following sections will involve a description of these techniques.

2.1.1 Light Microscopy.

Phase contrast light microscopy is generally used to examine those fibre samples collected in the occupational environment. Fibres are collected from the atmosphere via a filter head, or from water suspensions via filtration apparatus, onto the surface of a membrane filter. In order that the fibres may be observed optically, the filters must be dried, made transparent, and permanent preparations constructed. A method for this procedure has been adopted at the Institute of Occupational Medicine (IOM) in Edinburgh, following recommendations from the Asbestosis Research Council (ARC, 1971) and Australia NHMRC (1976). The system comprises the use of 0.8 μm pore Millipore filters, which may be cleared in acetone vapour and mounted in a drop of triacetin (glycerol triacetate) between a glass slide and a glass coverslip. This preparation technique involves little sample manipulation, thereby reducing the possiblity of fibre loss; it also provides a permanent preparation from which the sample can be quickly evaluated. Unfortunately, this technique has one major disadvantage, the majority of asbestos fibres are below the resolution of the light microscope (Ruud, 1978) and thus, only those fibres of greater than 0.3 μm diameter may be observed (Le Guen *et al.* 1980). It is essential, for the purposes of the present study, that the dimensions of the maximum possible number of fibres in an asbestos sample be estimated; thus it is evident that the light microscope would be of limited use in this study.

2.1.2 Scanning and Transmission Electron Microscopy Techniques.

The use of the SEM or TEM are by far the most accurate means of assessing fibre dimensions. The SEM can reputedly resolve fibres of 5 nm diameter and the TEM 0.2 nm (Chatfield, 1979); and whilst these figures would appear optimistic, they do illustrate that the TEM is capable of resolving samples which are one order of magnitude finer than may be observed using the SEM. The observation of fibres by SEM relies on differences in the surface characteristics of the sample for contrast against the background filter; a decrease in sample diameter, therefore, may mean that there

is not an adequate contrast for accurate visualisation of the fibres. This problem does not occur when the TEM is used, and it is therefore accepted that identification of very fine chrysotile fibrils may be more accurately carried out upon utilisation of the TEM (Chatfield., 1979).

The technique for sample preparation for SEM observation is considerably less complicated than the one employed for TEM observation. The preparation technique for SEM evaluation involves a direct transfer of the fibre coated membrane onto an SEM stub; the surface may be coated with a suitable agent such as gold, and directly examined. The types of filter used for SEM observation are either the Millipore cellulose ester membrane or the Nuclepore perforated polycarbonate membrane. The rough surface of the Millipore filter tends to obscure the smaller asbestos fibres, and the Nuclepore filter is therefore generally preferred for SEM observation (Ruud, 1978).

The preparation methods involved for TEM observation are more complicated due to the requirement that the filter be destroyed to allow passage of the electron beam. The filter material is removed by solvent dissolution using either chloroform for the Nuclepore membrane or acetone for the Millipore membrane, and this generally involves the incorporation of techniques based on the original work of Jaffe (1948). Cooke et al (1975) described a method in which the membrane filter is carbon coated, placed on top of an EM grid, and the filter dissolved using the Jaffe washer; a carbon replica of the filter surface as well as the fibre population is left on the EM grid, and this can then be examined. Organic materials or an unsuitable filter medium may also be removed by using an ashing procedure (Chatfield and Glass, 1976). The preparation of fibres for TEM observation would therefore appear to rely on a number of preparatory steps, and this may prove disadvantageous as it would be expected that the likelihood of either fibre breakage or loss occurring would increase as the preparatory procedure became more complicated. Another

disadvantage of TEM observation of fibres is the presence of bars on the EM grids, these effectively preclude the accurate assessment of any fibres that may be in contact with a bar.

The choice of technique for fibre assessment comprised the use of either the SEM or the TEM, and currently the use of the SEM would appear to be the most advantageous for the following reasons:

i) The technique for sample preparation for SEM observation requires relatively few steps compared to the TEM technique, and thus it would be expected that both fibre loss and breakage would be reduced.

ii) SEM observation of fibres also has the advantage that the whole of the surface of the filter can be examined, whereas TEM observation requires a restriction to those areas not covered by the grid bars. The use of the TEM would also encourage the inaccurate assessment of those longer fibres lying across the grid bars.

iii) Assuming that both preparation methods would prove reproducible, the SEM technique is faster, thereby saving both time and money, and this advantage requires some consideration when contemplating the preparation of a large number of samples.

On the basis of these advantages, it was decided that, for the purpose of this study, the SEM would be used for the estimation of fibre dimensions and fibre number for the available asbestos samples.

2.1.3 Aims and Objectives.

The aims of this study were to:

i) Establish a method using the SEM for accurate assessment of fibre dimensions and fibre number.

ii) Establish the reproducibility of this method.

iii) Establish the fibre number content for a variety of those asbestos samples which were to be examined in the cytotoxicity studies (Chapter 4), and also to assess the dimensions of a representative proportion of the fibres for each sample.

2.2 MATERIALS AND METHODS.

2.2.1 Fibre Collection or "Elutriation".

A number of different fibrous samples (Section 2.2.2) have been made available at the IOM for use in both *in vivo* and *in vitro* studies. The *in vivo* studies comprised the exposure of rats to dust by inhalation or via intratracheal or intraperitoneal routes of administration. Some of these samples were known to contain fibres that were outwith the respirable dimensions, and it became apparent that whilst the use of these samples in inhalation studies would result in the exposure of the lung to a respirable fraction of the sample, the utilization of the same material for intratracheal or intraperitoneal injection and also for *in vitro* studies would result in an exposure to non-respirable material. It was therefore decided that all of the fibrous samples would be passed through an "elutriation" process in order to collect the respirable portion of each sample. The elutriation apparatus has been described in detail by Beckett (1975); the dust was passed through a cyclone elutriator (based upon a design reported by Breuer (1964)); which effectively removed the majority of the non-respirable fibres (Beckett , 1975). The respirable fibrous samples were collected on a filter assembly placed between the dust generator and the animal exposure chamber. This method of collection of fibres has been used extensively at the IOM in order to ensure that the samples used in both *in vivo* and *in vitro* biological assay systems contained fibres of respirable dimensions (Wright *et al*, 1980; Bolton *et al*, 1982a). For the purpose of this thesis the abbreviation E will be used to indicate that a fibrous sample has passed through the elutriation process.

2.2.2 Types of Fibre Available for Examination and Their Source.

UICC Crocidolite, Amosite and Chrysotile A.

In 1966 samples of the main commercially important types of asbestos were prepared in Johannesburg by Timbrell (1969). The work was initiated by the Union Internationale Contre Le Cancer (UICC), in order to provide research groups with well-characterised, respirable samples of asbestos fibres. This resulted in the provision of a

set of standards so that direct inter-laboratory comparisons of results from biological experiments could be carried out. These samples have been used by the IOM as standards for *in vivo* and *in vitro* experiments.

E UICC Crocidolite, Amosite, Anthophyllite and Chrysotile A.

Elutriated versions of the UICC samples.

E Ceramic Fibre.

An industrial sample of ceramic aluminium silicate glass fibre (man-made) (Davis *et al*, 1982).

E Long Fibre (LF) Amosite and Short Fibre (SF) Amosite.

Both amosite samples were made available by a Canadian source, and SF amosite was prepared by grinding the long fibre version in water.

E Tremolite.

An elutriated version of Korean tremolite.

E Brucite.

An elutriated version of the fibrous form of $Mg(OH)_2$ (brucite).

SFA Chrysotile.

A sample of superfine chrysotile generously donated by the Medical Research Council (MRC) Pneumoconiosis Research Unit (PRU) at Penarth, Cardiff. This was originally prepared by sedimentation of a fully milled commercial sample of a Grade 7 chrysotile sample (Wagner and Berry, 1973).

E Parent of Heated (E PH) Chrysotile and E Heated (E H) Chrysotile.

E H chrysotile was heated to 850 °C in a dry heat oven; the parent material was a commercially available material.

E Factory (E F) Amosite and E Factory (E F) Chrysotile.

Both of these samples were obtained from the air filtration systems of asbestos factories.

Wet-Dispersed Chrysotile (WDC).

The preparation of WDC is an industrial technique described by Heron and Huggett (1971). In summary, the process comprises the use of a surface-active soap solution (wetting agent) which allows the chrysotile sample to disperse into fibrils and form a slurry. The slurry is passed through a fine nozzle and exposed to electrolytes which remove the soaps and encourage the fibres to bond firmly together. The resulting strands of WDC may be spun and woven to form an asbestos textile. The industrial process has involved the development of a chrysotile textile comprising individual fibrils that are tightly bonded together, and this has resulted in a considerable reduction in the generation of respirable fibres in the factory environment during the preparation procedure (Schneider, 1972). It would be expected that a reduction in the airborne respirable fibres would also involve a reduction in the health hazards normally associated with chrysotile, and an examination of the pathogenicity of this dust is therefore of particular importance. With this view in mind, a number of samples of WDC obtained from various stages of the WDC preparation process at a WDC factory preparation plant have been made available by the Asbestosis Research Council, in order that their respective pathogenicities be assessed.

a) E WDC - an elutriated version of the finished WDC product.
b) E Milled WDC - a textile yarn from the factory WDC process, the yarn was milled to generate dust for experimental purposes.
c) E Milled Chrysotile - a sample prepared by milling a standard chrysotile textile yarn made from the same type of chrysotile as used in the WDC process.
d) E Unextracted WDC - a sample prepared as in b), but from which the detergent had not been removed.
e) E Heat-Cleaned WDC - a sample prepared as in b) but heated to 300 °C for 16 hrs to remove the detergent.
f) E Factory WDC - a sample collected from the factory environment at the plant producing sample b).

2.2.3 Development of Methods and Validation of Assay System.

In order to assess the dimensions and fibre number content of the above fibrous samples a technique was employed which is described in detail in Section 2.2.4. In brief this technique comprised the filtration of a known weight of fibres onto a Nuclepore filter; the fibres were then coated in gold and observed using the SEM. This method involved the initial use of a 0.4 μm pore filter, as recommended by Beckett (1973), but this procedure was later repeated using a 0.2 μm pore filter in order to reduce the loss of small and fine fibres. A number of experimental replicates were established to test the reproducibility and accuracy of the preparation procedure and these will be described in detail in the following section.

2.2.3.1 Preliminary Experiments Using 0.4 μm Pore Filters.

One filter was prepared for each fibrous sample, and the lengths and diameters of 200 fibres for each filter were measured using the SEM. In order to assess the reproducibility of the preparation procedure, samples of UICC crocidolite and E PH chrysotile were prepared in duplicate and 200 fibres from each filter were assessed. A technique using ultrasound was employed to disperse the fibrous samples prior to filtration (Section 2.2.4.2); in order to assess any damage to the fibres due to their exposure to ultrasound a number of samples of unsonicated UICC material were prepared by shaking the fibres in water prior to filtration. The fibre dimensions for each sample were examined and compared with the dimensions for ultrasonicated material.

2.2.3.2 Experiments Using 0.2 μm Pore Filters.

The technique described in Section 2.2.4 was employed to prepare the fibrous samples for SEM observatiom, and 0.2 μm pore filters were used in an attempt to reduce fibre loss through the pores. In order to assess the accuracy and reproducibility of the preparation procedure, ie dilution and filtration of the samples, a number of filters were prepared from one original 10 mg sample of

UICC amosite. These comprised two filters containing 5 μg of dust, one filter of 2.5 μg and one filter of 10 μg. The dimensions of 100 fibres together with the number of fields required to count the 100 fibres were recorded. In order to assess the reproducibility of the author's fibre counting method, as well as the even dispersal of fibres on the filter, 100 fibres together with the number of fields required to count them were measured using a different area from each filter. To evaluate the reproducibility of both the weighing and ultrasonication techniques, the above procedure was repeated using a separate 10 mg sample of amosite.

It has been reported by Beckett and Attfield (1974), in connection with fibre counting using the light microscope, that considerable differences between individual counters and also laboratories can occur, even when the observers assess the same specimen. As this situation may also apply to the SEM, the reliability of the author's counting on the SEM was checked by inviting one of the "routine fibre counters" from the IOM to assess one of the author's replicate amosite samples.

Following these preliminary experiments, duplicate filters from all of the available fibrous samples were prepared according to the methods described in Sections 2.2.4.1 - 2.2.4.4. The dimensions of 100 fibres from each filter were assessed and the statistical analyses of the data is described in Section 2.2.5. In addition, the number of fields required to count 100 fibres was assessed, and this procedure was repeated for five different areas on each filter. The data was used to calculate the fibre number content for each sample, the treatment of the data is described in Section 2.2.5.2.

2.2.4 Technical Procedure for the Preparation of Fibre Samples for the Estimation of Fibre Dimensions and Fibre Number by SEM.

In order that the fibre dimensions and fibre number content of the various samples of asbestos should be assessed by SEM, it was necessary that a number of accurate and reproducible procedures be employed for the preparation of these samples, and these will

be described in the following sections. A preliminary study in which 0.4 μm pore Nuclepore filters were employed was carried out, and at a later stage the 0.2 μm pore filters were utilised instead. The preparation and counting procedures used for each type of filter will be described in this section.

2.2.4.1 Weighing of Fibre Samples.

10.0 mg of each fibrous sample were weighed onto an aluminium weighing boat using a Beckman LM 800 balance. Each 10 mg sample was transferred into a plastic 25 ml Universal container (Sterilin) for storage. Care was taken during the transferral of samples from the boat to the container that electrostatic charges did not cause fibre loss by forcing fibres backwards and out of the tube.

2.2.4.2 Dispersal, Dilution and Filtration of Fibre Samples.

10.0 mg of each fibrous sample were suspended in 10.0 mls of pre-filtered (0.2 μm pore Nuclepore) distilled, deionised water. All dilutions were obtained using a range of automatic liquid dispensers (Gilson Pipettman) which were found to have an accuracy of 0.15%. The fibre suspensions were ultrasonicated for 2 mins at 100 KHz in a Contes ultrasonic cleaner bath to encourage the disaggregation and even dispersal of the fibres throughout the suspensions. All fibrous samples were ultrasonicated unless otherwise stated.

All samples were further diluted to give a concentration of 10 μg/ml by transferring 0.1 mls of suspended fibres into 9.9 mls of water. A new pipette tip was used between samples, and each tip was immersed for 30 secs in the new fibre sample to allow maximum adherence of the fibres to the plastic surface, thereby reducing the loss of fibres from the ultimate sample due to adherence to the tip. An aliquot of fibre suspension containing a known weight of fibres was transferred via a pipette onto a 25 mm Nuclepore filter (of either 0.2 or 0.4 μm pore size, Section 2.2.4), with the shiny surface of the filter facing upwards. Millipore filtration apparatus was used for all experiments and thoroughly cleansed in between different fibre samples. A 0.4 μm pore backing filter was used

to encourage even distibution of the fibres across the filter area and also to prevent the passage of fibres through the continuous filter pores. Upon occasions, random samples of the filtered fluid were refiltered onto 0.2 μm pore Nuclepore filters to look for fibre loss.

2.2.4.3 Preparation of Filters for SEM Observation.

Each filter was cut in half and mounted, using double-sided Sellotape, onto a 13 mm aluminium SEM stub (Agar Aids). The excess filter was trimmed to the edge of the stub and the edges were painted with Quick Drying Silver Paint (Agar Aids) to ensure electrical contact with the stub. The completed preparations were allowed to dry and coated with an approximately 250 Å thick layer of gold using a Nanotech SEM Prep 2 sputter coater.

2.2.4.4 Preparation of the SEM for Observation of Fibres.

All fibres were examined using a Cambridge Instruments S600 SEM at a magnification of 10,000 x. The specimen height was altered so that a 1 cm measured length on the TV screen would be equivalent to 1 μm actual length. The SEM was adjusted to give optimum performance and the fibres observed on the TV screen using a "slow scan" raster speed, as recommended by Middleton (1982), rather than the faster TV scan speed, as this system presented a clearer picture of the fibres on the TV screen.

2.2.4.5 Counting Rules for the Assessment of Fibre Dimensions.

The counting rules employed in this study to ascertain the dimension distribution of a fibrous sample were those currently in use at the IOM. A fibre, for the purpose of this thesis, was defined, and therefore recorded if it possessed an aspect ratio of greater than 3:1 (ARC Technical Note 1, 1971). The lengths and diameters of those fibres observed in a field of view were measured using a clear plastic ruler graded in mm. Any curly chrysotile fibre that was observed was measured along the equivalent of its straightened length (Walton, 1982). In order that the bias towards counting the

longer fibres in a sample be eliminated, only those fibres whose ends entered the top or right-hand side of the field, or were completely contained within the field were counted, as recommended by Schneider (1978). Should a fibre prove too long to be measured in one field of view at 10,000 x, the magnification was decreased. If a clump of fibres was observed within the field of view, attempts were made to measure the fibres within the clump. However, if several large clumps were observed on the stub, thereby suggesting a poor distribution of fibres across the filter, the sample was discarded and a new one prepared. If any fibres appeared in very close apposition to each other, the position of contact was examined at a higher magnification to ascertain if these fibres were indeed separate or if they were the result of the splitting of an end of one fibre. This proved particularly important in the case of chrysotile where the ends appeared frayed. Any non-fibrous particles that were found in association with or attached to fibres were ignored.

The lengths and diameters were recorded for 200 fibres per stub for 0.4 μm pore filters, and 100 fibres per stub for the 0.2 μm pore filters. Fields of view were selected randomly by moving one of the stage manipulator rods in consecutive half turns along a straight line acrosss the filter. Only those fibres that complied with the prescribed counting rules were recorded. If, at any time, the field of view required refocussing, the specimen height was correspondingly altered so that 1 cm was again equivalent to 1 μm actual length.

2.2.4.6 Counting Rules for the Assessment of Fibre Number.

In order to assess the fibre number content of an asbestos sample, the number of fields required to count 100 fibres was noted at five random points on each duplicate stub. The same counting rules were applied as are described in Sections 2.2.4.4 and 2.2.4.5.

2.2.5 The Presentation and Statistical Analysis of the Fibre Data.

A number of sets of data were available following the preparation procedure described in Section 2.2.3:

0.4 μm pore filter:
i) Dimensions of 200 fibres for all samples as a result of assessing one stub.
ii) Dimensions of 200 fibres for each of two replicates for UICC crocidolite and E PH chrysotile.
iii) Dimensions of 200 fibres for samples of unsonicated UICC crocidolite , amosite and chrysotile.

0.2 μm pore filter:
i) Dimensions of 100 fibres for the 10 μg (one filter), 5 μg (two filters), and 2.5 μg (one filter) samples that had been prepared from one 10 mg sample, together with the number of fields required to count the fibres.
ii) Repeat of i) using the same stub.
iii) Repeat of i) and ii) using a separate 10 mg sample.
iv) Dimensions of 100 fibres for the two replicate filters prepared for each fibrous sample, together with the number of fields required to count 100 fibres at five separate points on each filter.

2.2.5.1 Treatment and Statistical Analysis of Fibre Dimension Data.

The length and diameter measurements for each population of fibres were sorted into defined groups as shown in Tables 2.1 and 2.2. The number and thus the percentage of fibres to fall within each defined group was recorded. The data was finally expressed in terms of the percentage of fibres greater than each stated length or diameter present in each fibre population. The data was presented in a tabulated form, or alternatively in a graphical form on "log 2 cycles x probability" paper (Chartwell).

In order to determine the statistical difference between the various fibre length and diameter distributions, a Kolmogorov-Smirnov two-sample test analysis was used (Siegel, 1956). In the case

Table 2.1 Defined Length Groups

Abbreviations for Group µm	Lengths Included Within Group µm
0	0 - < 1
1	1 - < 2
2	2 - < 3
3	3 - < 4
4	4 - < 5
5	5 - < 6
6	6 - < 7
7	7 - < 8
8	8 - < 9
9	9 - < 10
10	10 - < 15
15	15 - < 20
20	20 - < 30
30	30 - < 40
40	40 - < 50
50	50 - < 100
100	100 +

Table 2.2 Defined Diameter Groups

Diameter Group µm
0.1 (ie. 0 - 0.1)
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0

onwards.

of the data from the 0.2 μm pore filters, if no statistically significant difference was observed between the fibre length distributions from the two replicates, the two sets of data were amalgamated to form a final count of 200 fibres per fibrous sample.

The Kolmogorov-Smirnov Analysis.

This is a test which is used to determine whether two independent samples have been drawn from the same population or from populations with the same distribution, and is sensitive to any difference in distribution. The two-sample test is concerned with agreement between the two sets of sample values. If the samples have been drawn from the same population distribution then the cumulative distributions of both samples would be expected to be similar. However, should the two sample cumulative distributions prove to be "too far apart" at any point, this would suggest that the samples have come from different populations. A suitable deviation between the two sample cumulative distributions is evidence for rejecting the possibility that they came from the same population. The following steps summarise the procedure for the Kolmogorov-Smirnov two-sample test (Siegel, 1956):

1) Arrange each of the two groups of scores in cumulative frequency distribution using the same interval for each.
2) By subtraction determine the difference between the two sample cumulative distributions at each listed point.
3) Determine the largest of these differences - D.
4) For a 2-tailed test, when n_1 and n_2 are larger than 40, Table 2.3 is used. It is not necessary that n_1 be equal to n_2. Critical values of D for any given large values of n_1 and n_2 may be computed from Table 2.3. For this study n_1 and n_2 are generally equal to either 100 or 200.
5) Upon comparison of two fibre length distributions a level of $p<0.05$ was considered to be statistically significant.

2.2.5.2 Treatment and Statistical Analysis of Fibre Number Data.

A mean figure was obtained from the five sets of data available concerning the number of fields required to count 100 fibres from one

Table 2.3 Kolmogorov-Smirnov Two-Sample Test

Table of Critical Value of D

Significance Level	Value of D so large as to call for rejection at the indicated level of significance	Maximum D value for $n_1+n_2=100$	Maximum D value for $n_1+n_2=200$
.1	$1.22\sqrt{\frac{n_1+n_2}{n_1\ n_2}}$	17.3	12.2
.05*	$1.36\sqrt{\frac{n_1+n_2}{n_1\ n_2}}$	19.2	13.6
.025	$1.48\sqrt{\frac{n_1+n_2}{n_1\ n_2}}$	20.9	14.8

*Level of significance used at IOM.

Adapted from Siegel (1956)

stub. The Student T Test (Bailey, 1974) was used to compare the mean figures for the two replicate filters from each fibrous sample. If no significant difference was found between the two sets of data, the results were amalgamated and used to calculate the fibre number content for each sample.

Calculation of Fibre Number.

Filter area = 183.9 mm^2.
Effective SEM field area = 9.99×10^{-5} mm^2.
Thus there are 1.84×10^{6} field areas/filter area.
5 µg of fibres were filtered onto the membrane area, thus 2.7×10^{-6} µg of fibres were available on one field area.

$$\text{Number of fibres/ } \mu g = \frac{1}{\frac{2.7 \times 10^{-6}}{\text{mean no. fibres/field}}}$$

The results were expressed in terms of number of fibres/10^{-10} g.

2.3 RESULTS.

2.3.1 The Morphological Appearance of Fibrous Dust Samples.

The morphological appearance of a selection of the fibrous dusts are shown in Figures.2.1 to.2.11. It can be seen that UICC crocidolite, UICC amosite, E tremolite, E UICC anthophyllite and also E brucite (Figures 2.1, 2.2, 2.4, 2.5, 2.6) are straight in appearance, whereas the UICC chrysotile and also SFA chrysotile (Figures 2.3 and 2.10) samples possess a number of curly fibres. The E LF amosite sample (Figure 2.7) contained a large proportion of long and straight fibres compared to the SF amosite offspring (Figures 2.8 and 2.9) which comprised relatively short material. The E PH, and E H chrysotile and also E F amosite and chrysotile samples are not illustrated here, but possessed the expected morphological appearance of straight fibres in the amosite samples and a proportion of curly fibres in the chrysotile samples; in addition all four types were contaminated with non-fibrous material. The SFA chrysotile sample (Figure 2.10), despite its suggestive nomenclature, did not appear to contain fibres that were finer than those seen in the UICC chrysotile sample. The WDC (Figure 2.11) proved to be the asbestos sample with the finest fibres; all of these fibres were extremely long and fine in nature, and also interwoven, thus making an assessment of the fibre length distribution for this and other WDC samples impossible.

2.3.2 Results Obtained Using 0.4 μm Pore Filter.

A number of preliminary experiments were carried out using 0.4 μm pore Nuclepore filters to assess fibre dimensions; these comprised an examination of the reproducibility of the preparation technique, the assessment of a selection of the fibrous samples, and an examination of any alterations in fibre length distribution due to the use of the ultrasonic bath.

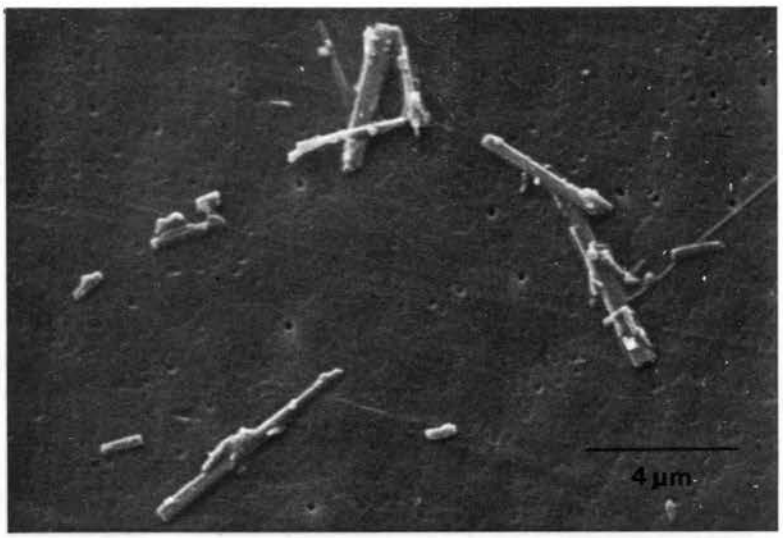

Figure 2.1 SEM Photograph of UICC Crocidolite (x 5000).

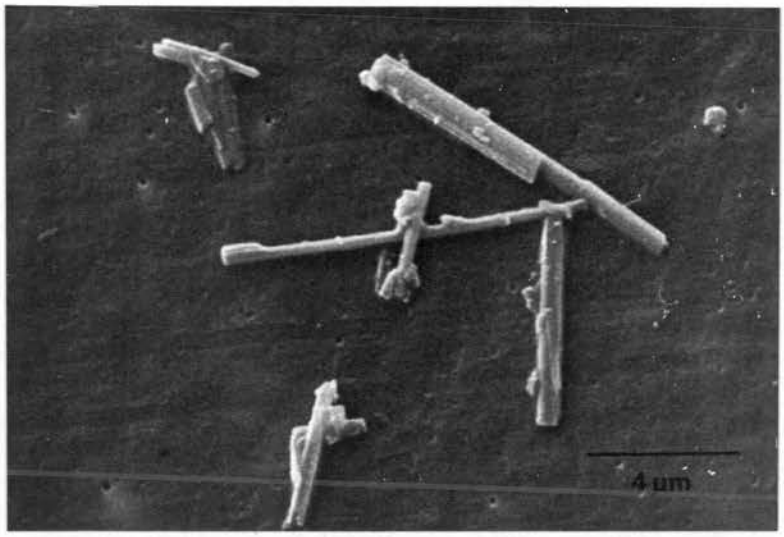

Figure 2.2 SEM Photograph of UICC Amosite (x 5000).

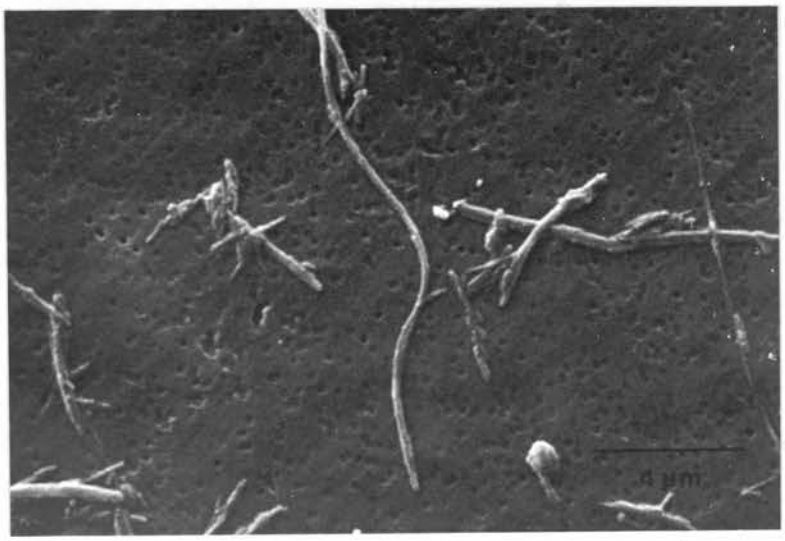

Figure 2.3 SEM Photograph of UICC Chrysotile (x 5000).

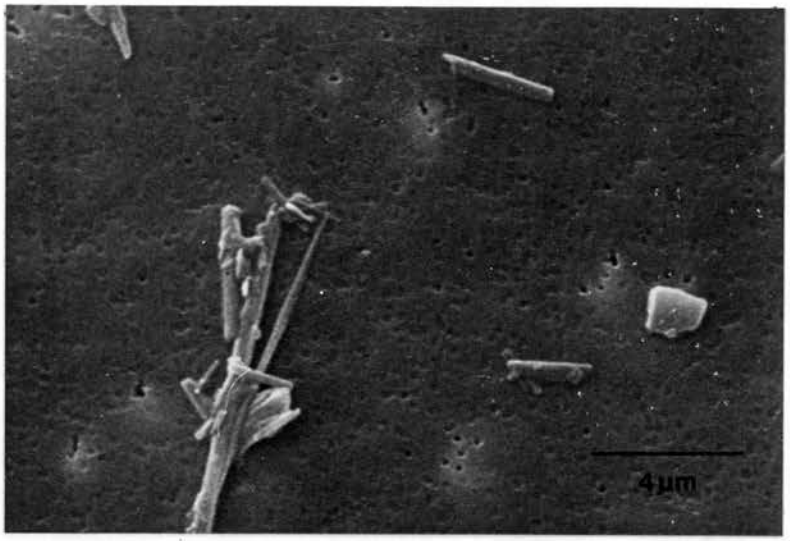

Figure 2.4 SEM Photograph of E Tremolite (x 5000).

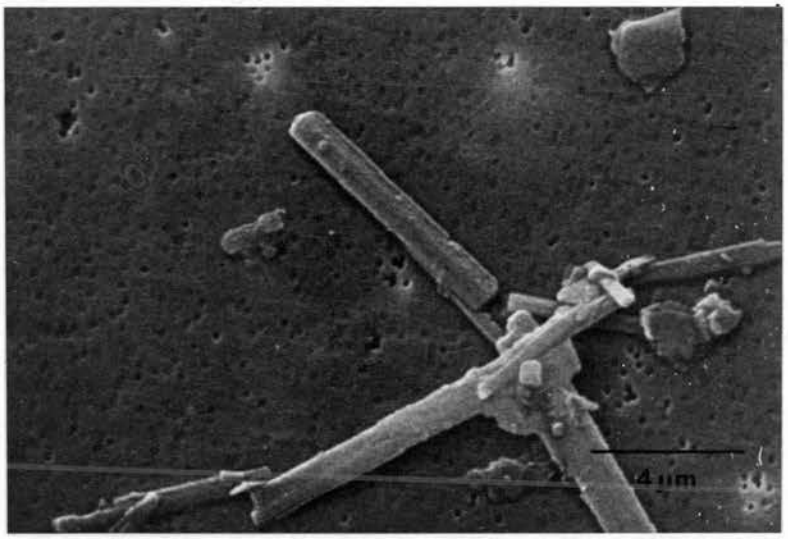

Figure 2.5 SEM Photograph of E UICC Anthophyllite (x 5000).

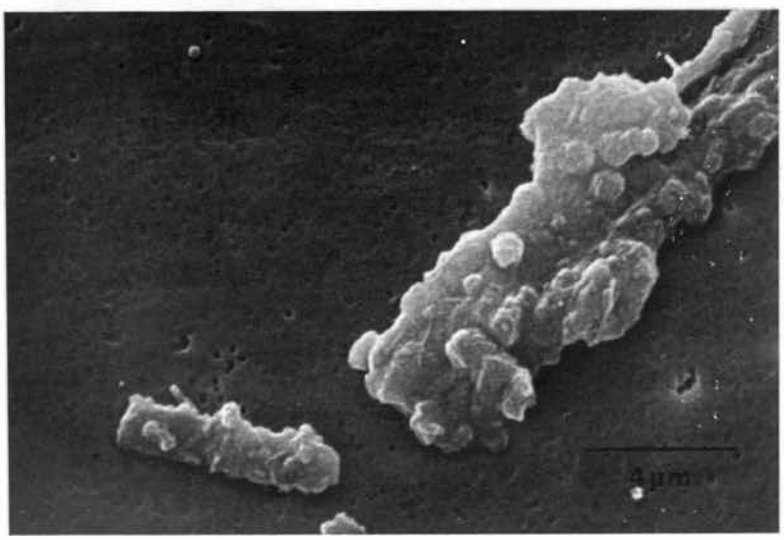

Figure 2.6 SEM Photograph of E Brucite (x 5000).

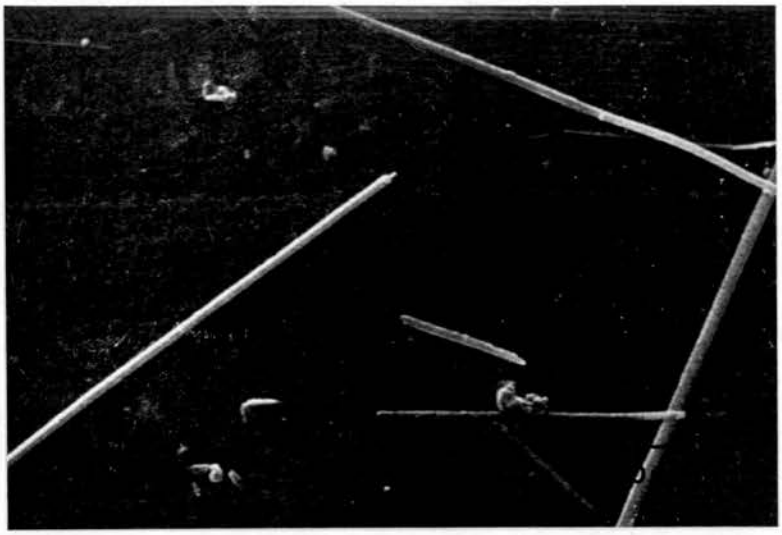

Figure 2.7 SEM Photograph of E LF Amosite (x 2000).

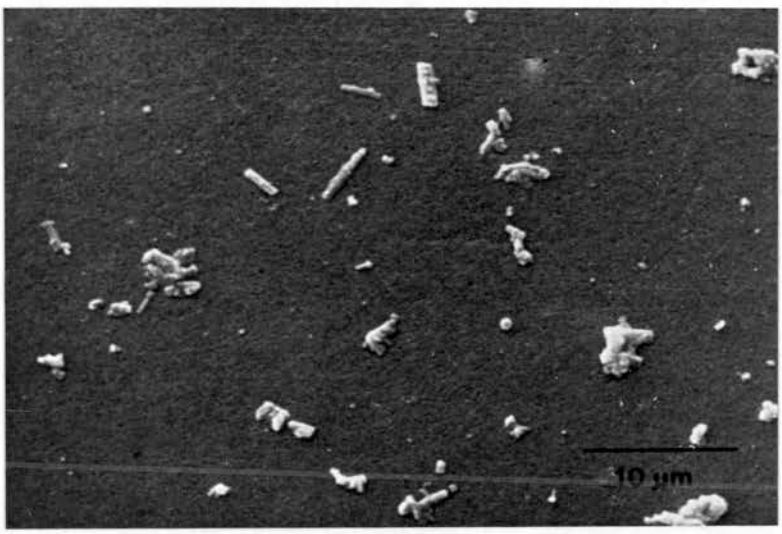

Figure 2.8 SEM Photograph of SF Amosite (x 2000).

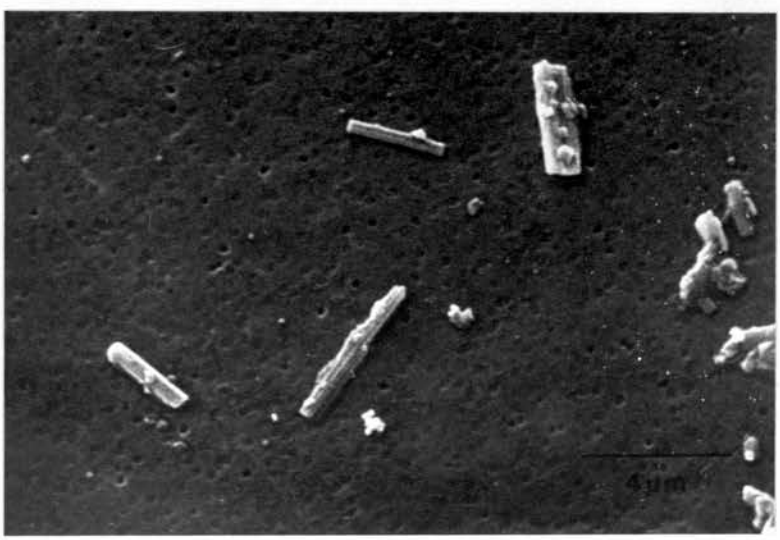

Figure 2.9 SEM Photograph of SF Amosite (x 5000).

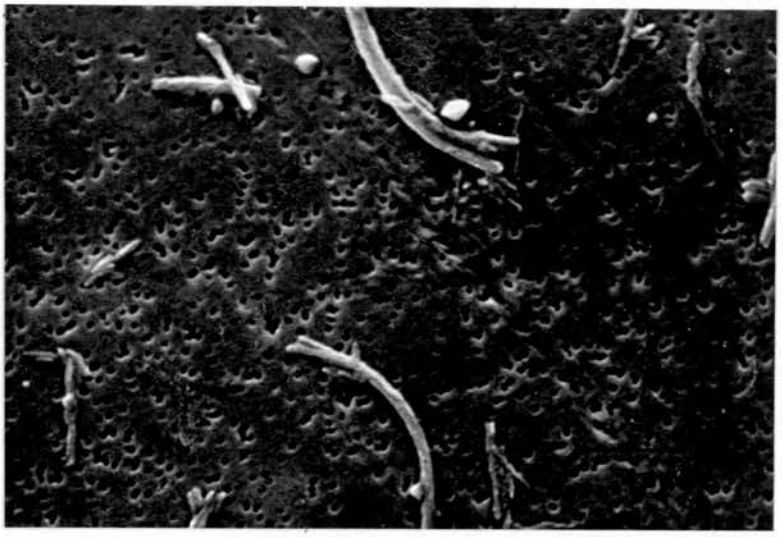

Figure 2.10 SEM Photograph of SFA Chrysotile (x 5000).

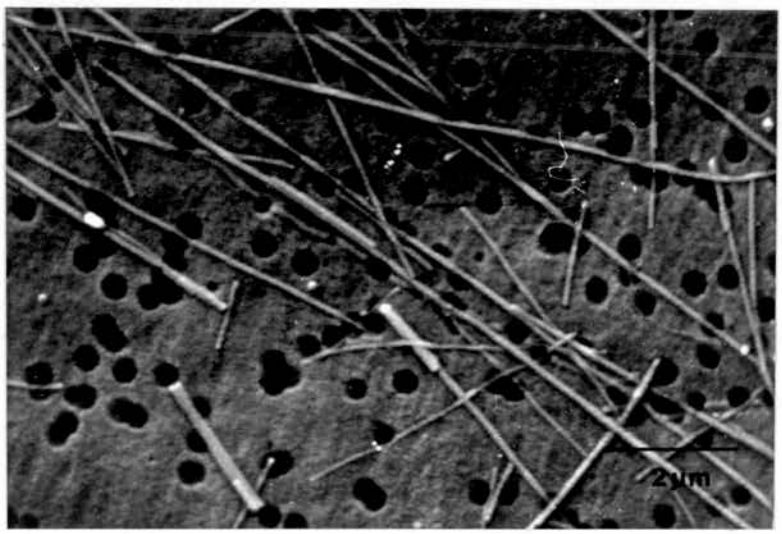

Figure 2.11 SEM Photograph of E WDC (x 10,000).

2.3.2.1 The Reproducibility of the Preparation Technique.

The fibre length distributions for the duplicate preparations of UICC crocidolite and E PH chrysotile are shown in Table 2.4 i) and ii). No statistical difference was found between either of the two sets of replicate data, and no difference was observed between the types of fibre.

2.3.2.2 The Length and Diameter Distributions for the Fibrous Samples.

The fibre length and diameter distributions for a number of the fibrous samples are shown in Tables 2.5 and 2.6 and Figures 2.12 to 2.14. Of the UICC samples, chrysotile would appear to contain the largest number of fine fibres as only 48% of the chrysotile fibres are greater than 0.3 μm in diameter. The elutriation process appeared to have an effect on both UICC amosite and chrysotile (Tables 2.5 and Figures 2.12 and 2.13); E UICC amosite possessed a greater number of shorter fibres than the non-elutriated UICC material ($p<0.01$) although the diameter distribution was not significantly altered; E UICC chrysotile had a similar fibre length distribution, but possessed a greater number of finer fibres than the non-elutriated material ($p<0.01$).

For the factory samples, E F amosite showed a similar fibre length distribution to E UICC amosite (Figure 2.12) although the sample contained a greater number of finer fibres of less than 0.4 μm ($p<0.01$). E F chrysotile possessed a larger number of long and thick fibres compared to the UICC material ($p<0.01$) (Figure 2.13, Tables 2.6 and 2.7). Heating the parent chrysotile sample had the effect of reducing the number of shorter and fine fibres in the sample ($p<0.01$) (Figure 2.14, Table 2.6). The length distribution for the SFA chrysotile sample proved similar to the UICC chrysotile sample (Figure 2.13) although a greater number of finer fibres was observed. E ceramic fibre possessed a proportion of fibres that were relatively long and thick compared to the other samples (Figure 2.14, Table 2.6), the mean diameter being approximately 0.6 μm.

Table 2.4 Reproducibility of Preparation Technique Using 0.4 μm Pore Filter.

i) Cumulative Fibre Length Distributions for UICC Crocidolite.

Fibre length μm	Replicate number "1"	"2"
0	100	100
1	90	95
2	49	52
3	25	29
4	15	18
5	11	10
6	9	6
7	7	2
8	5	1
9	3	1
10	2	1
15	1	-

ii) Cumulative Fibre Length Distributions for E PH Chrysotile.

Fibre Length μm	Replicate number "1"	"2"
0	100	100
1	93	90
2	48	53
3	30	27
4	17	16
5	13	14
6	9	12
7	6	8
8	4	7
9	4	5
10	3	4
15	2	-
20	1	-

Table 2.5 Cumulative Fibre Length and Diameter Distributions Using 0.4 μm Pore Filter.

	UICC crocidolite	UICC amosite	UICC chrysotile A	E UICC amosite	E UICC chrysotile A
Length μm					
0	100	100	100	100	100
1	90	99	87	96	93
2	49	84	50	63	56
3	25	64	31	40	38
4	15	52	18	25	26
5	11	46	13	20	19
6	9	37	10	13	13
7	7	30	8	8	10
8	5	26	6	7	9
9	3	22	4	4	7
10	2	20	3	3	6
15	1	10	2	1	2
20	-	6	2	1	1
30	-	4	1	-	1
40	-	2	-	-	-
50	-	1	-	-	-
Diameter μm					
.1	100	100	100	100	100
.2	99	100	98	99	90
.3	67	84	48	78	25
.4	27	64	5	55	16
.5	10	25	-	21	7
.6	5	19	-	14	3
.7	2	11	-	9	2
.8	1	9	-	5	1
.9	-	8	-	4	-
1.0	-	6	-	2	-
>1.0	-	4	-	-	-

Table 2.6 Cumulative Fibre Length and Diameter Distributions Using 0.4 μm Pore Filter Continued.

	E factory amosite	E factory chrysotile	E PH chrysotile	E H chrysotile	SFA chrysotile	E ceramic
Length μm						
0	100	100	100	100	100	100
1	90	99	93	97	75	100
2	63	72	48	72	43	63
3	39	48	30	52	30	30
4	26	36	17	37	17	16
5	17	27	13	28	12	12
6	12	21	9	22	9	9
7	7	15	6	13	5	6
8	7	12	4	9	5	5
9	6	8	4	8	3	4
10	4	5	3	7	2	4
15	3	2	2	3	-	2
20	2	1	1	2	-	2
30	2	-	-	2	-	2
40	2	-	-	1	-	2
50	2	-	-	-	-	1
100	1	-	-	-	-	1
Diameter μm						
.1	100	100	100	100	100	100
.2	100	89	95	100	41	100
.3	78	43	52	94	7	100
.4	36	31	22	70	2	96
.5	23	22	10	42	2	68
.6	17	14	6	25	2	45
.7	11	13	3	15	2	16
.8	8	11	3	11	2	13
.9	5	10	3	7	1	11
1.0	5	9	2	4	-	11
>1.0	2	8	1	4	-	7

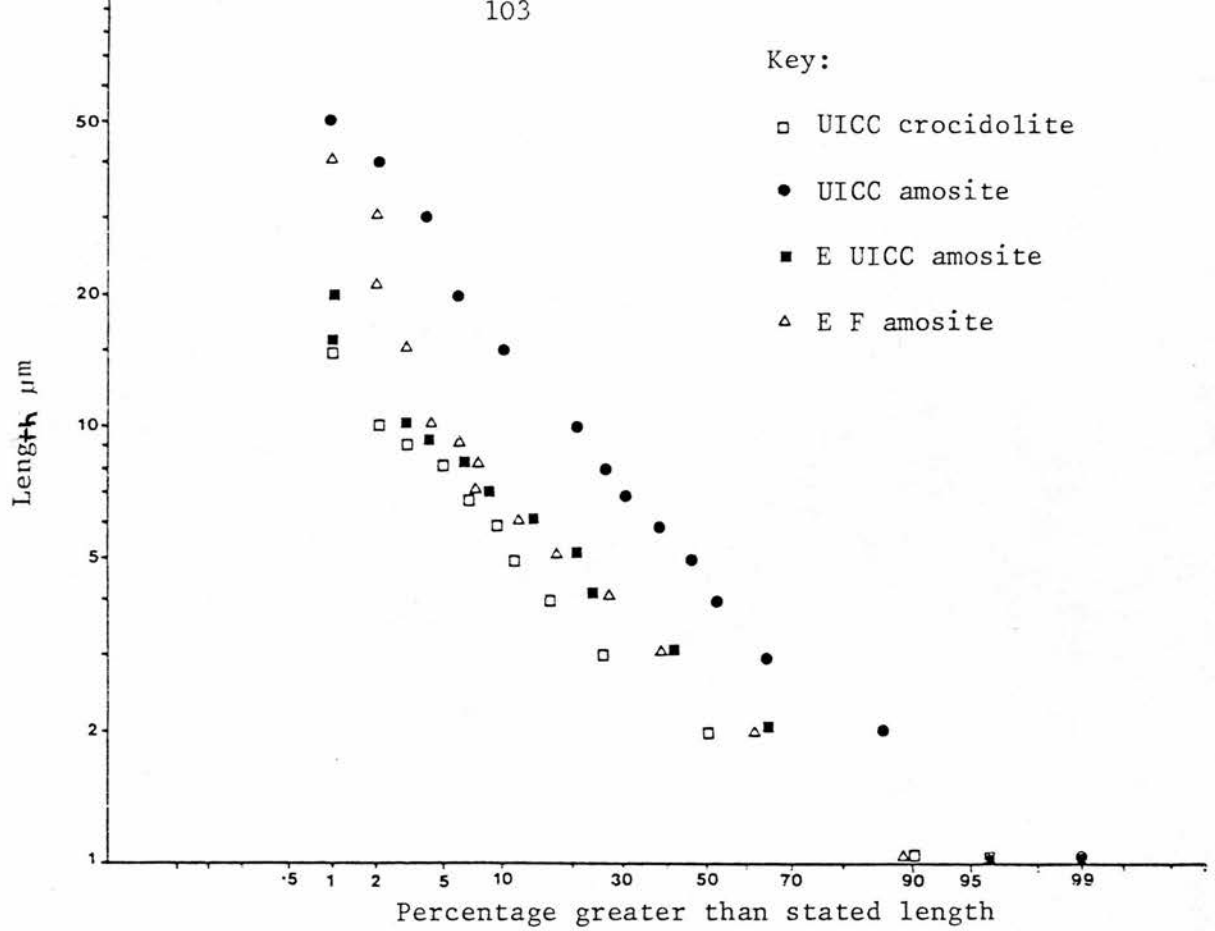

Figure 2.12 Fibre Length Distributions for UICC Crocidolite, UICC Amosite, E UICC Amosite, E F Amosite Using 0.4 µm Pore Filter.

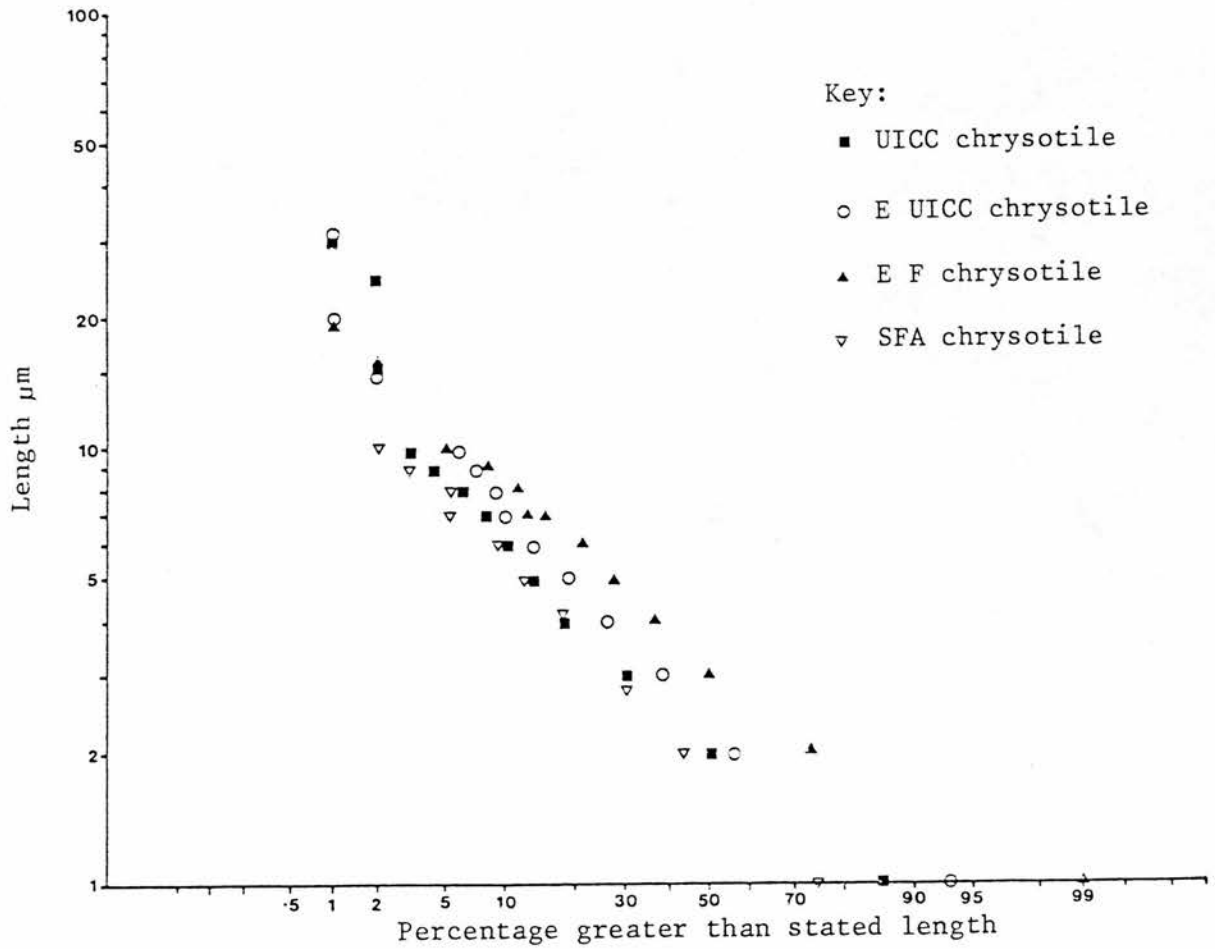

Figure 2.13 Fibre Length Distributions for UICC Chrysotile, E UICC Chrysotile, E F Chrysotile, SFA Chrysotile Using 0.4 µm Pore Filter.

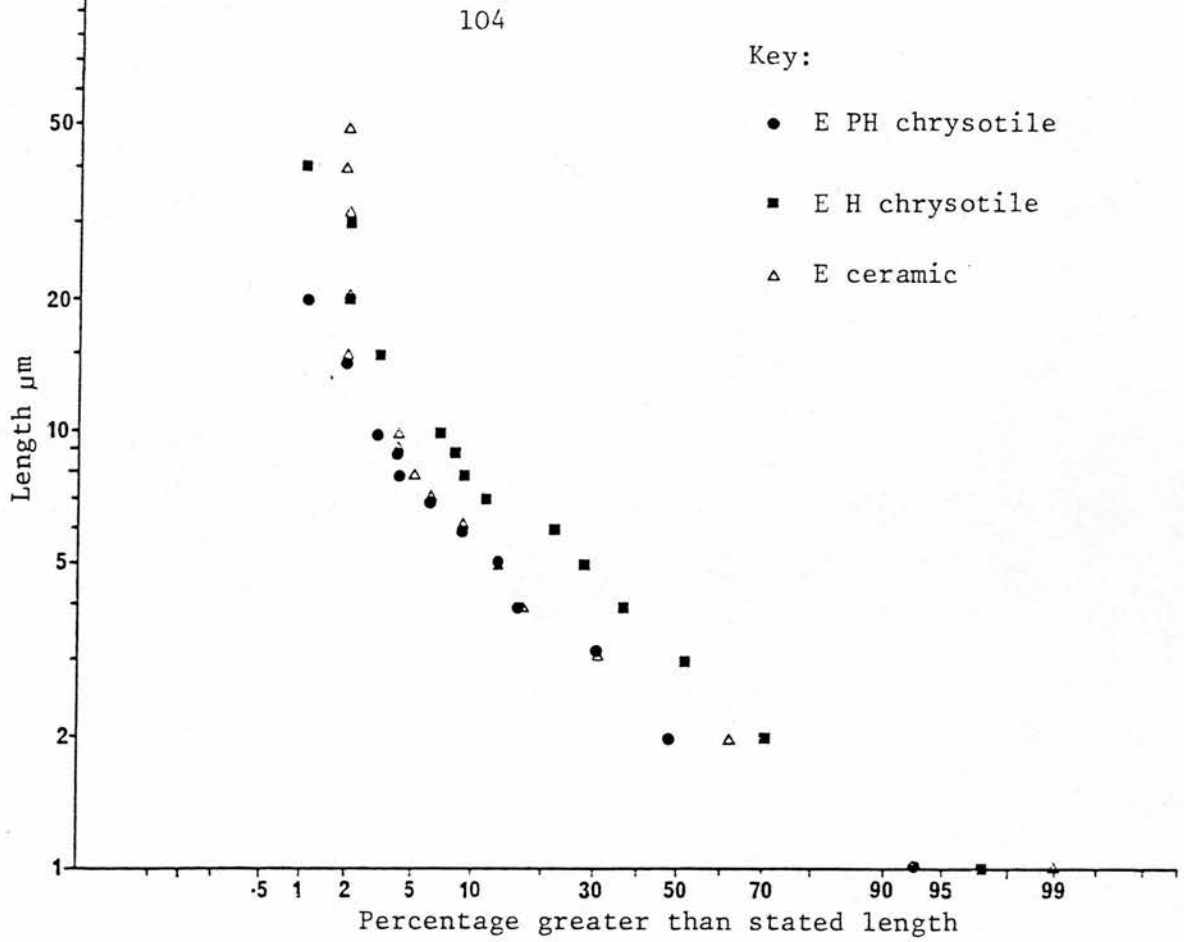

Figure 2.14 Fibre Length Distributions for E PH Chrysotile, E H Chrysotile and E Ceramic Using 0.4 µm Pore Filter.

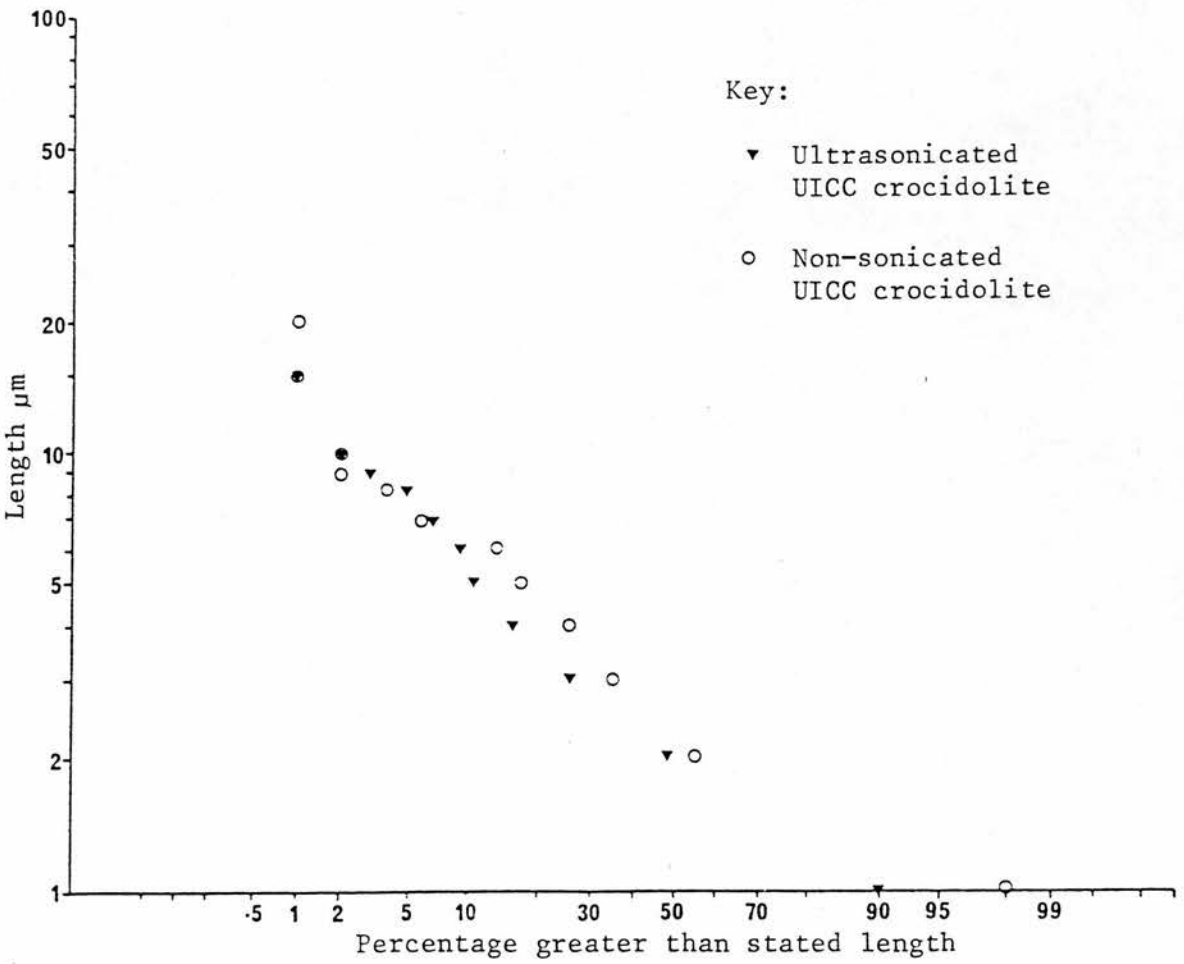

Figure 2.15 Fibre Length Distributions for Ultrasonicated and Non-Sonicated UICC Crocidolite Using 0.4 µm Pore Filter.

It did not prove possible to assess the fibre length distributions for the E WDC samples for the majority of the fibres were very fine ie. 0.1 μm diameter and less (Figure 2.11), and also long and intertwined. Some of the fibres were so long, that at a magnification of 10,000x they proved to be longer than one field of view; it was not possible, however, to accurately measure such fine fibres at a magnification of less than 10,000x on the available SEM. The interwoven nature of the fibres also increased the difficulty experienced in evaluation of fibre length. For these reasons, no further measurements were attempted on any of the other WDC samples.

A number of samples were not examined using the 0.4 μm pore Nuclepore filters because they were not available at the appropriate preparation time, these samples were E UICC crocidolite, E UICC anthophyllite, E tremolite, E brucite, E LF amosite and SF amosite. It should be noted that for all of the other samples examined on the 0.4 μm pore filters, very few fibres of less than 1 μm in length were observed.

2.3.2.3 The Effect of Ultrasonication of the UICC Samples.

The fibre length and diameter distributions for the non-sonicated UICC samples are shown in Table 2.7. When these figures are compared with results for the sonicated samples given in Table 2.5 (Figures 2.15, 2.16 and 2.17), it can be seen that ultrasonication had no significant effect upon the crocidolite sample (Figure 2.15), ultrasonication of amosite (Figure 2.16), however significantly reduced the number of smaller and finer fibres ($p<0.01$). The overall fibre length distribution for UICC chrysotile was not significantly affected by ultrasonication (Figure 2.17), although the number of fibres of finer diameter was significantly reduced ($p<0.01$). It would therefore appear, from these results, that the ultrasonication procedure may have an effect upon the length and/or diameter distribution of a fibrous sample, and this may vary depending upon the type of fibre involved.

Table 2.7 Cumulative Fibre Length and Diameter Distributions for "Unsonicated" UICC Asbestos Samples Using 0.4 μm Pore Filter.

	UICC crocidolite	UICC amosite	UICC chrysotile A
Length μm			
0	100	100	100
1	98	95	78
2	55	71	44
3	36	50	28
4	26	39	19
5	17	29	13
6	13	19	7
7	6	15	7
8	4	12	6
9	2	10	4
10	2	9	2
15	1	5	-
20	1	4	-
30	-	3	-
40	-	2	-
50	-	2	-
>100	-	1	-
Diameter μm			
.1	100	100	100
.2	95	100	67
.3	57	74	19
.4	29	46	4
.5	12	22	2
.6	7	11	1
.7	3	8	1
.8	2	4	1
.9	2	3	1
1.0	2	2	1
>1.0	1	1	1

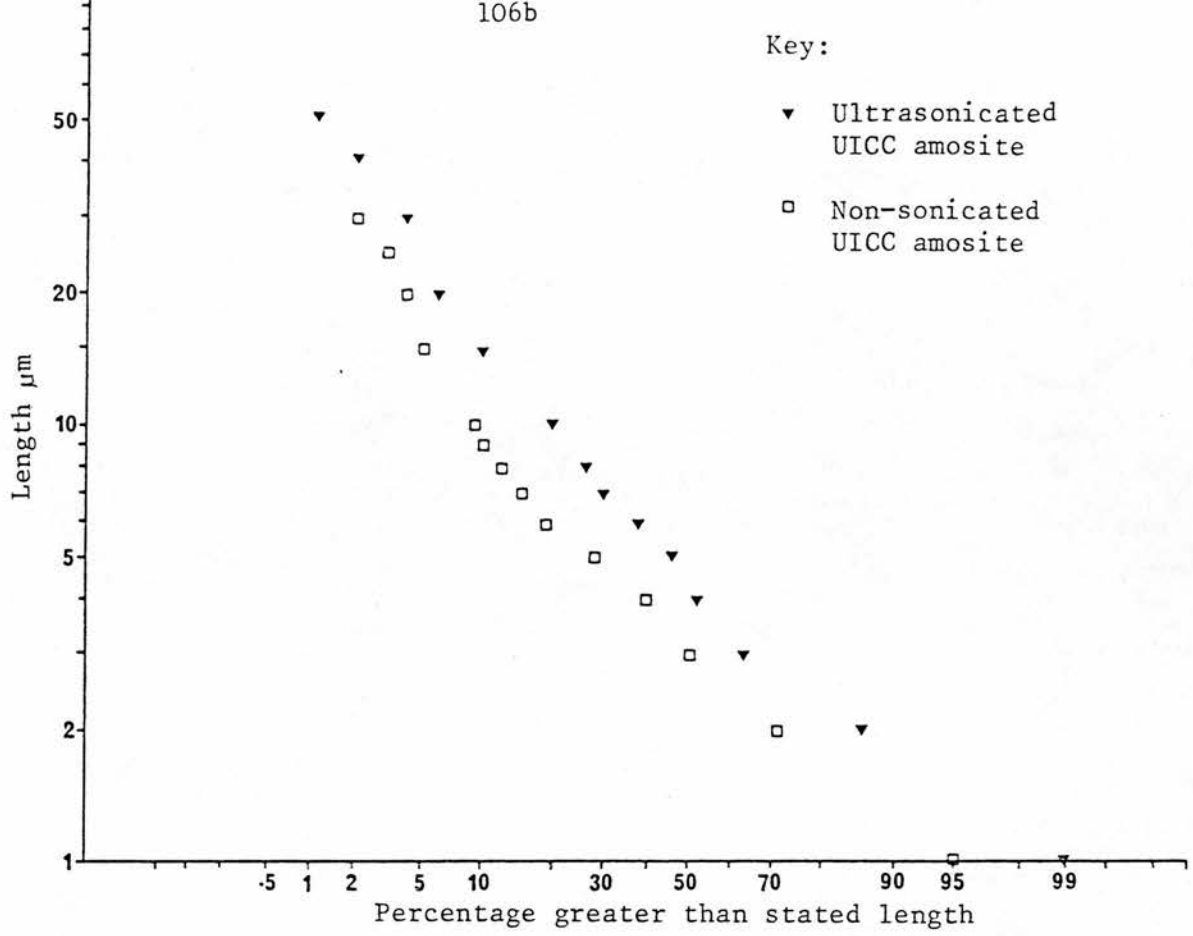

Figure 2.16 Fibre Length Distributions for Ultrasonicated and Non-Sonicated UICC Amosite Using 0.4 µm Pore Filter.

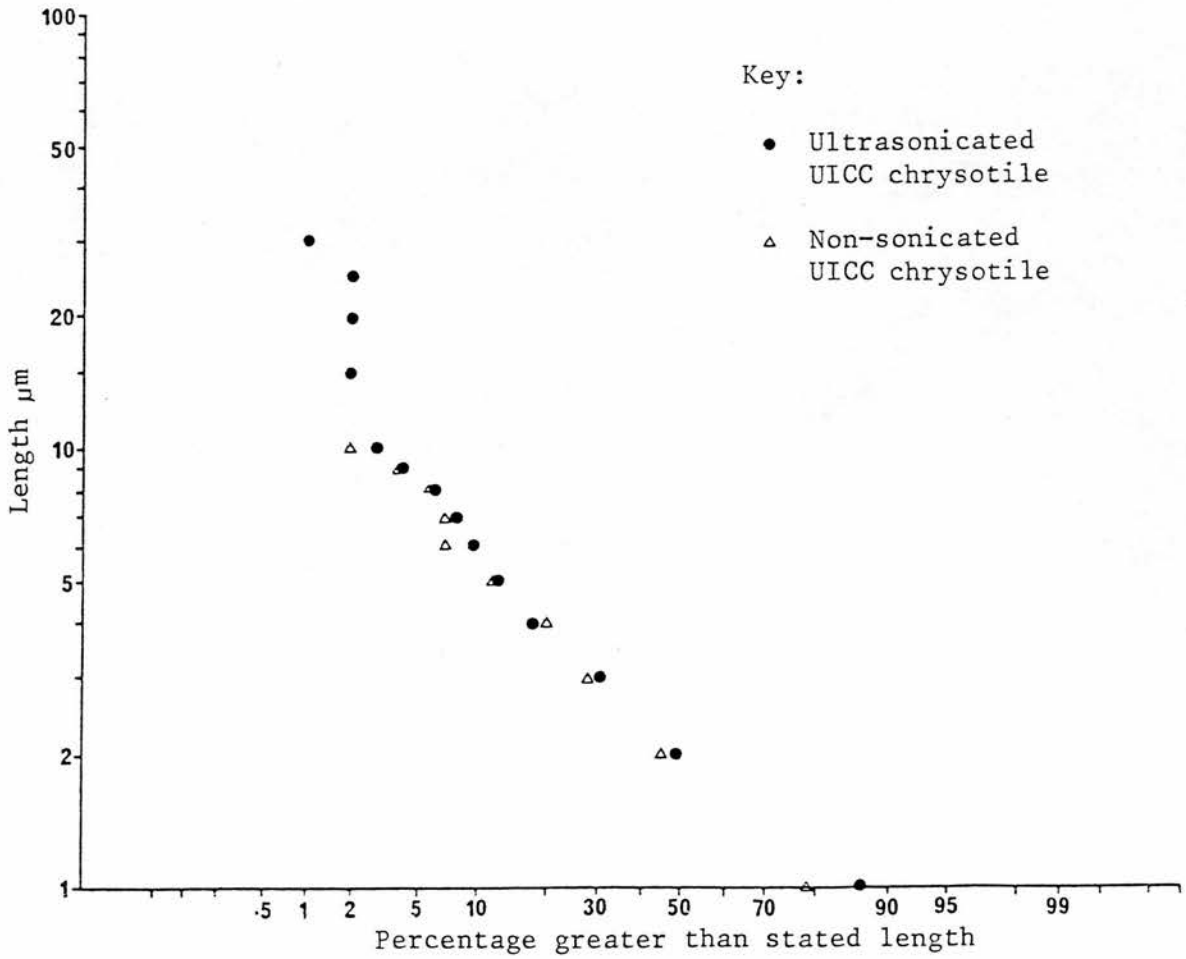

Figure 2.17 Fibre Length Distributions for Ultrasonicated and Non-Sonicated UICC Chrysotile Using 0.4 µm Pore Filter.

2.3.3 Results Obtained Using 0.2 μm Pore Nuclepore Filters.

The 0.2 μm pore Nuclepore filters were employed for the final assessment of both the length and diameter distributions of all of the fibrous samples. Prior to the assessment of these samples, however, a number of replicates were prepared from two UICC amosite samples, to assess the reproducibility of both the preparation and the counting procedure.

2.3.3.1 The Assessment of the Reproducibility of the Preparation Techniques Using UICC Amosite Replicates.

The fibre length distributions for a number of replicate filters bearing various known weights of UICC amosite are shown in Table 2.8, the corresponding diameter distribution in Table 2.9, and the number of fields required to count 100 fibres in Table 2.10. Using the Kolmogorov-Smirnov analysis, all of the fibre length distributions were shown to be drawn from the same population of fibres, with the exception of amosite preparation 1, 5 μg (2) count (1), and this sample proved significantly different from amosite preparations number 2, 5 μg (1) (1) and number 2, 5 μg (1) (2) ($p<0.01$). No significant difference was observed between the replicate counts for each individual filter. With regard to the diameter distributions (Table 2.9) all of the samples were shown to be drawn from the same original population of fibres.

The number of fields required to count 100 fibres per filter (Table 2.10) proved reproducible, standard deviations of 5.8, 4.5 and 2.1 were obtained for the means of the 5 μg, 2.5 μg, and 10 μg data respectively. The 5 μg weight proved to be a convenient concentration for counting on the SEM , as this filtered weight allowed the presence of a maximum number of fibres per field area, but encouraged the formation of only a minimum number of clumps.

2.3.3.2 The Reproducibility of Inter-Observer Counting.

A comparison of the author's assessment of fibre length and diameter distributions together with the results obtained by an experienced counting person from the IOM are shown in Table 2.11.

Table 2.8 Reproducibility of Preparation Technique Using 0.2 μm Pore Filter and UICC Amosite Replicates : Cumulative Fibre Length Distributions.

Length μm	UICC amosite preparation no 1								Weight of amosite and filter no. Count no
	5 μg (1)		5 μg (2)		2.5 μg		10 μg		
	1	2	*1	2	1	2	1	2	
0	100	100	100	100	100	100	100	-	
1	88	91	91	91	86	81	74	-	
2	51	42	58	54	45	50	45	-	
3	29	29	40	23	27	29	25	-	
4	15	15	25	14	19	20	14	-	
5	9	7	15	11	11	14	7	-	
6	7	4	12	5	8	10	7	-	
7	5	3	10	5	5	9	6	-	
8	3	2	6	3	5	5	4	-	
9	3	2	6	3	4	2	4	-	
10	1	1	4	3	2	2	2	-	
15	-	-	2	2	1	1	1	-	
20	-	-	1	1	1	1	1	-	
30	-	-	1	1	-	1	1	-	
40	-	-	-	1	-	-	-	-	
Length μm	UICC amosite preparation no 2								Weight of amosite and filter no. Count no
	5 μg (1)		5 μg (2)		2.5 μg		10 μg		
	*1	*2	1	2	1	2	1	2	
0	100	100	100	100	100	100	100	100	
1	81	85	81	83	87	83	82	83	
2	37	38	38	44	45	52	45	45	
3	19	18	23	29	27	27	27	25	
4	11	13	10	14	19	12	18	17	
5	9	5	8	7	14	9	13	13	
6	7	5	6	6	11	6	10	10	
7	5	4	3	4	8	4	10	8	
8	5	3	3	2	6	4	8	7	
9	5	1	1	-	5	3	8	7	
10	5	-	1	-	2	2	6	6	
15	1	-	1	-	1	1	1	2	
20	1	-	-	-	-	1	1	-	
30	1	-	-	-	-	-	-	-	
40	-	-	-	-	-	-	-	-	

* $p<0.01$ for significant differences between preparation 1:5 μg (2)(1) and preparation 2: 5 μg (1)(1) and 5 μg (1)(2).

Table 2.9 Reproducibility of Preparation Technique Using 0.2 μm Pore Filter and UICC Amosite Replicates : Cumulative Diameter Distributions.

Diameter μm	UICC amosite preparation no 1								Weight of amosite and filter no. Count no
	5 μg (1)		5 μg (2)		2.5 μg		10 μg		
	1	2	1	2	1	2	1	2	
.1	100	100	100	100	100	100	100	-	
.2	86	92	89	92	90	83	84	-	
.3	50	58	59	56	52	52	53	-	
.4	39	37	44	33	37	38	34	-	
.5	22	24	32	20	20	21	21	-	
.6	12	8	18	11	9	12	12	-	
.7	8	6	9	6	7	4	8	-	
.8	6	4	5	3	5	2	2	-	
.9	5	3	4	2	4	1	1	-	
1.0	1	-	2	2	1	-	1	-	
>1.0	-	-	1	2	1	-	1	-	

Diameter μm	UICC amosite preparation no 2								Weight of amosite and filter no. Count no
	5 μg (1)		5 μg (2)		2.5 μg		10 μg		
	1	2	1	2	1	2	1	2	
.1	100	100	100	100	100	100	100	100	
.2	84	80	84	80	86	85	79	86	
.3	44	54	53	44	54	47	46	57	
.4	26	35	30	26	31	23	27	37	
.5	16	19	14	20	17	14	17	19	
.6	7	10	8	15	6	7	11	10	
.7	4	4	5	8	3	2	8	3	
.8	4	3	2	4	2	2	3	2	
.9	3	2	1	3	2	2	2	2	
1.0	2	2	1	-	1	2	1	2	
>1.0	2	-	1	-	1	1	1	1	

Table 2.10 Reproducibility of Preparation Technique Using 0.2 μm Pore Filter and UICC Amosite Replicates : Field Count.

Preparation no.	Weight of amosite and filter no.	Count no.	No of fields required to count 100 fibres
UICC amosite no 1	5 μg (1)	1	38
	"	2	48
	5 μg (2)	1	56
	"	2	57
	2.5 μg	1	91
	"	2	86
	10 μg	1	23
	"	2	-
UICC amosite no 2	5 μg (1)	1	44
	"	2	52
	5 μg (2)	1	50
	"	2	50
	2.5 μg	1	98
	"	2	95
	10 μg	1	18
	"	2	21

Table 2.11 Comparison of Author's (AW) Counts With Experienced IOM Fibre Counter (JC).

Length μm	Cumulative Fibre Length Distribution	
	Author	IOM Fibre Counter
0	100	100
1	88	74
2	51	40
3	29	22
4	15	15
5	9	11
6	7	6
7	5	4
8	3	4
9	3	3
10	1	3
15	-	1
Diameter μm	Cumulative Diameter Distribution	
	Author	IOM Fibre Counter
.1	100	100
.2	86	91
.3	50	61
.4	39	43
.5	22	29
.6	12	15
.7	8	12
.8	6	5
.9	5	4
1.0	1	3

No significant difference was observed between the two sets of data for either fibre length or diameter distributions.

2.3.3.3 The Fibre Length and Diameter Distributions for the Fibrous Samples.

The fibre length and diameter distributions for those samples examined using a 0.2 μm pore filter are shown in Tables 2.12 to 2.14, and a selection of these are expressed graphically in Figures 2.18 to 2.23. The results have been produced from an amalgamation of the counts for the two separate filters prepared for each sample.

In the case of the UICC samples (Table 2.12), crocidolite possessed the greatest number of shorter fibres ie. 75% less than 2 μm ($p<0.01$ between samples of UICC crocidolite and chrysotile); amosite, however, had a larger number of thick fibres at 46% greater than 0.3 μm ($p<0.01$ between UICC amosite and either crocidolite or chrysotile). Elutriation of the UICC samples had no significant effect upon either the fibre length or diameter distributions (Table 2.12, Figures 2.18, 2.19 and 2.20) of the samples.

The E LF amosite sample proved considerably longer than its short fibre derivative ($p<0.01$) and comprised 31% of fibres greater than 5 μm in length, whereas the majority of SF amosite fibres were below 1 μm in length (Table 2.13, Figure 2.21). E brucite (Table 2.13, Figure 2.22) comprised many small and fine fibres, whereas E tremolite and E UICC anthophyllite were relatively long and thick by comparison ($p<0.01$).

The fibre length distribution for SFA chrysotile (Figure 2.20) proved similar to the one seen for the UICC chrysotile sample, although a considerably larger proportion of fibres of less than 0.2 μm diameter was observed ($p<0.01$) ie. 76% less than 0.2 μm versus 45% for UICC chrysotile (Tables 2.12 and 2.14). The fibre length distribution for the E H chrysotile did not differ significantly from that of the parent, (Table 2.14, Figure 2.23), although the number of fibres with diameters of less than 0.2 μm was significantly reduced ($p<0.01$).

Table 2.12 Cumulative Fibre Length and Diameter Distributions Using 0.2 μm Pore Filter.

	UICC crocidolite	UICC amosite	UICC chrysotile A	E UICC crocidolite	E UICC amosite	E UICC chrysotile A
Length μm						
0	100	100	100	100	100	100
1	67	78	76.5	66	77	71.5
2	25.5	35.5	43.5	24.5	37	33.5
3	12.5	24	26	9.5	21	17
4	7	15	14	6.5	12.5	7.5
5	4	9.5	10	4	7.5	5
6	3	7.5	6.5	2.5	4	4
7	2	4	5.5	1.5	2	3
8	1.5	3	4	0.5	2	3
9	1	1.5	3	0.5	0.5	1.5
10	1	1.5	2.5	0.5	0.5	1.5
15	-	-	0.5	-	-	1
20	-	-	0.5	-	-	0.5
30	-	-	0.5	-	-	0.5
40	-	-	0.5	-	-	0.5
50	-	-	0.5	-	-	0.5
100	-	-	0.5	-	-	-
Diameter μm						
.1	100	100	100	100	100	100
.2	63.5	81	55	65.5	75	52
.3	22	46.5	19	21.5	39	18.5
.4	8.5	26	8.5	8	27	7.5
.5	3.5	11.5	2	3	14.5	3.5
.6	0.5	8	-	2	8	1.5
.7	0.5	4.5	-	1.5	3	0.5
.8	-	1.5	-	1	1	0.5
.9	-	1.5	-	1	0.5	0.5
1.0	-	0.5	-	-	-	-
>1.0	-	0.5	-	-	-	-

Table 2.13 Cumulative Fibre Length and Diameter Distributions Using 0.2 μm Pore Filter (Continued).

	SF amosite	E LF amosite	E brucite	E tremolite	E UICC anthophyllite
Length μm					
0	100	100	100	100	100
1	44	87	56.5	87	83.5
2	8.5	73	30	56	56.5
3	3	59	18	36.5	42.5
4	0.5	37	13	27	30.5
5	0.5	31	8.5	17.5	25
6	0.5	24	7	11	18.5
7	-	22	6	7.5	15
8	-	20	6	7	10
9	-	19	5	6.5	7.5
10	-	17	4.5	5.5	6.5
15	-	12	1.5	2.5	2
20	-	10	0.5	1	1
30	-	5	-	1	0.5
40	-	1	-	0.5	0.5
50	-	-	-	-	0.5
100	-	-	-	-	-
Diameter μm					
.1	100	100	100	100	100
.2	57	71	48.5	91.5	88
.3	25.5	41	22	60.5	67.5
.4	12	28	16	38	52
.5	4	15	12.5	22.5	34
.6	1	8	8	16.5	27.5
.7	-	2	8	11	17
.8	-	-	8	7.5	14
.9	-	-	7	6	9.5
1.0	-	-	7	4	8
>1.0	-	-	6	1	6

Table 2.14 Cumulative Fibre Length and Diameter Distributions Using 0.2 μm Pore Filter (Continued).

	SFA chrysotile	E ceramic	E F amosite	E F chrysotile	E PH chrysotile	E H chrysotile
Length μm						
0	100	100	100	100	100	100
1	76	46	75.5	70	72	66.5
2	42	24	44.5	29	40	29
3	20	20	26.5	19.5	19.5	15
4	12	15	17	10.5	10.5	8
5	8	11	14	8	4	4
6	5	8	10	5.5	3	3
7	5	8	6.5	3.5	3	2.5
8	3	7	5	3	3	1.5
9	2	6	5	3	2	1
10	2	5	2.5	2.5	1.5	-
15	-	4	1	2	0.5	-
20	-	4	0.5	1	0.5	-
30	-	2	-	0.5	-	-
40	-	1	-	0.5	-	-
50	-	-	-	-	-	-
100	-	-	-	-	-	-
Diameter μm						
.1	100	100	100	100	100	100
.2	24	60	87.5	49.5	48	65
.3	6	27	48.5	16	11.5	24.5
.4	3.5	21	29	10	6	14
.5	1	15	17	6.5	3.5	7
.6	1	12	8.5	5	3	4.5
.7	-	10	4.5	4.5	2	3
.8	-	9	3.5	3.5	0.5	1.5
.9	-	8	3	3	0.5	0.5
1.0	-	7	2.0	2	0.5	0.5
>1.0	-	5	1.5	2	-	-

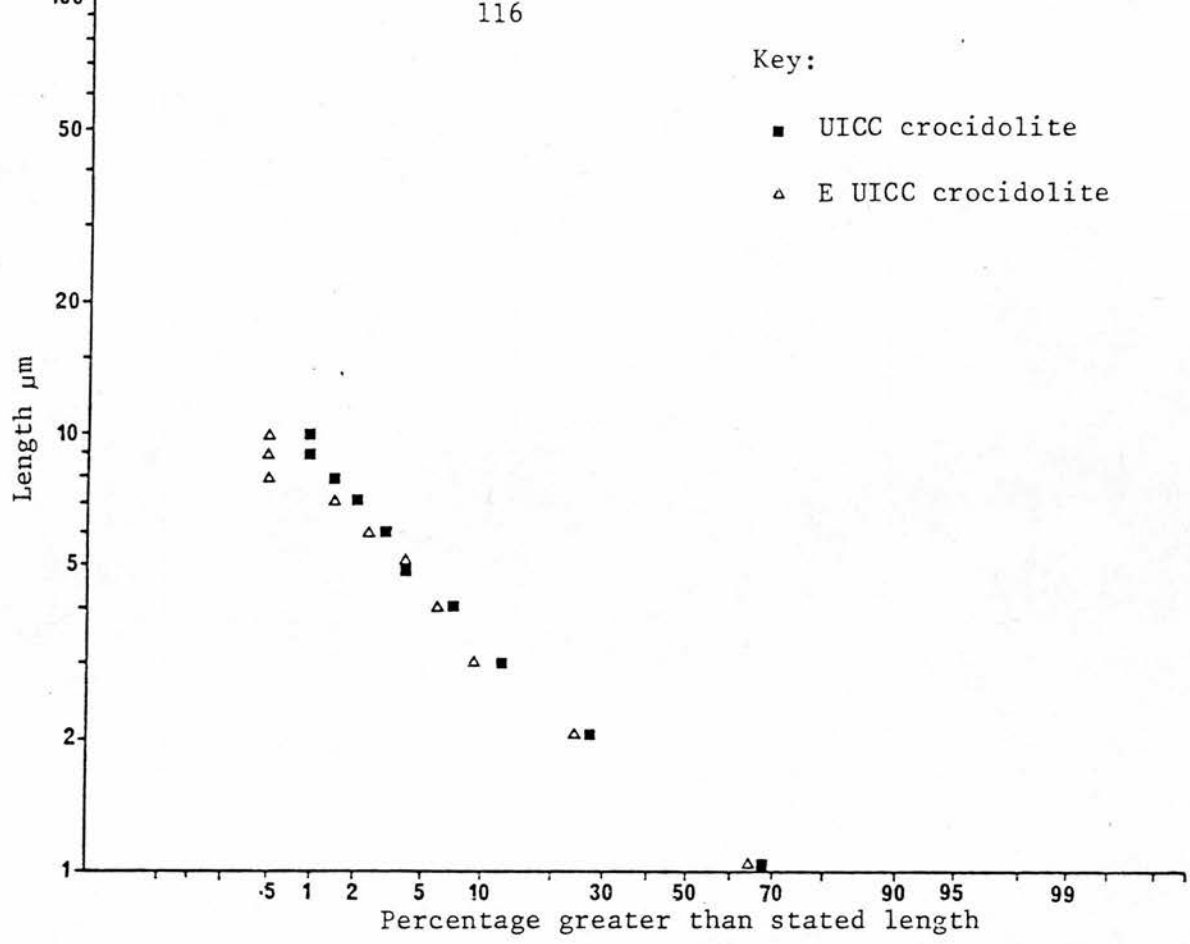

Figure 2.18 Fibre Length Distributions for UICC Crocidolite and E UICC Crocidolit Using 0.2 µm Pore Filter.

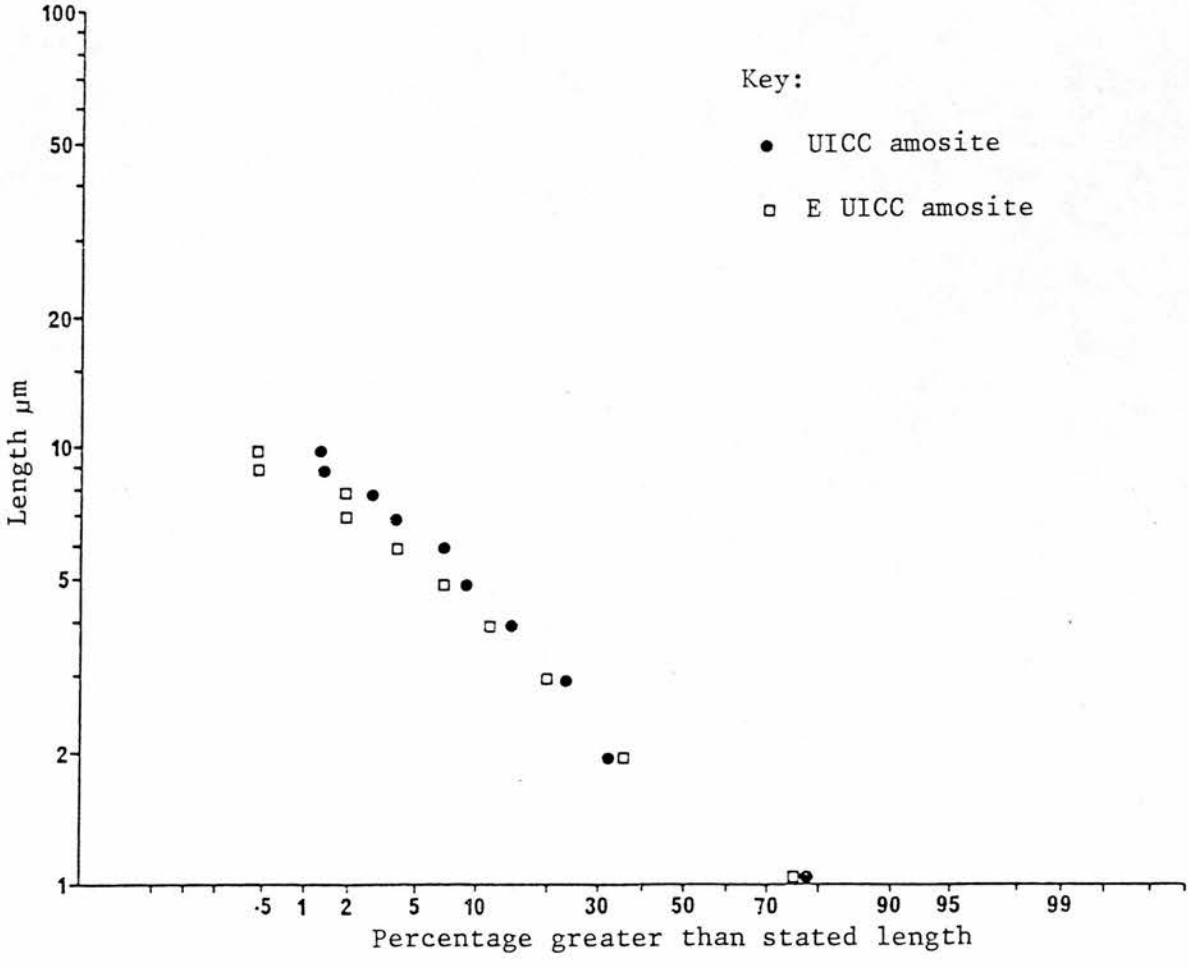

Figure 2.19 Fibre Length Distributions for UICC Amosite and E UICC Amosite Using 0.2 µm Pore Filter.

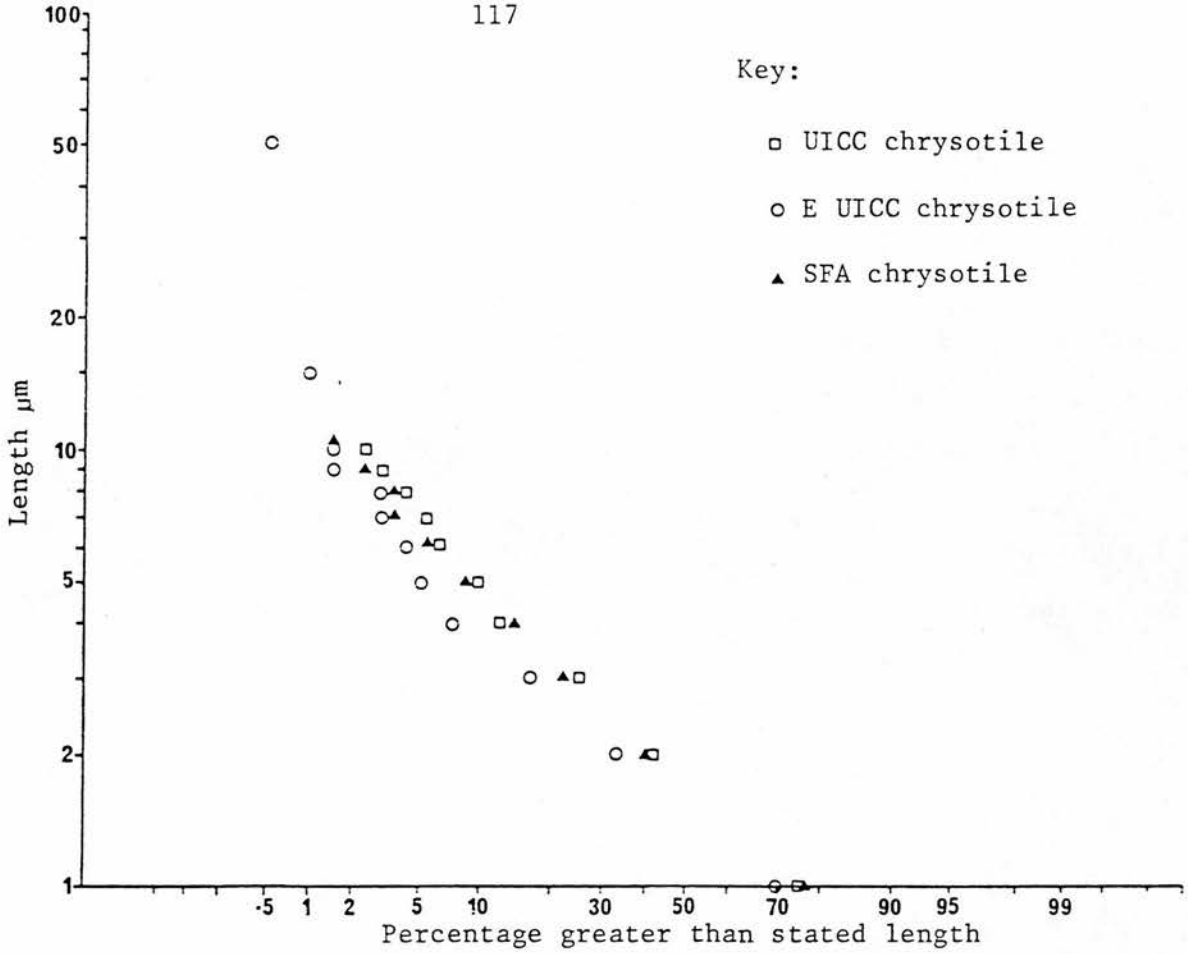

Figure 2.20 Fibre Length Distributions for UICC Chrysotile, E UICC Chrysotile and SFA Chrysotile Using 0.2 µm Pore Filter.

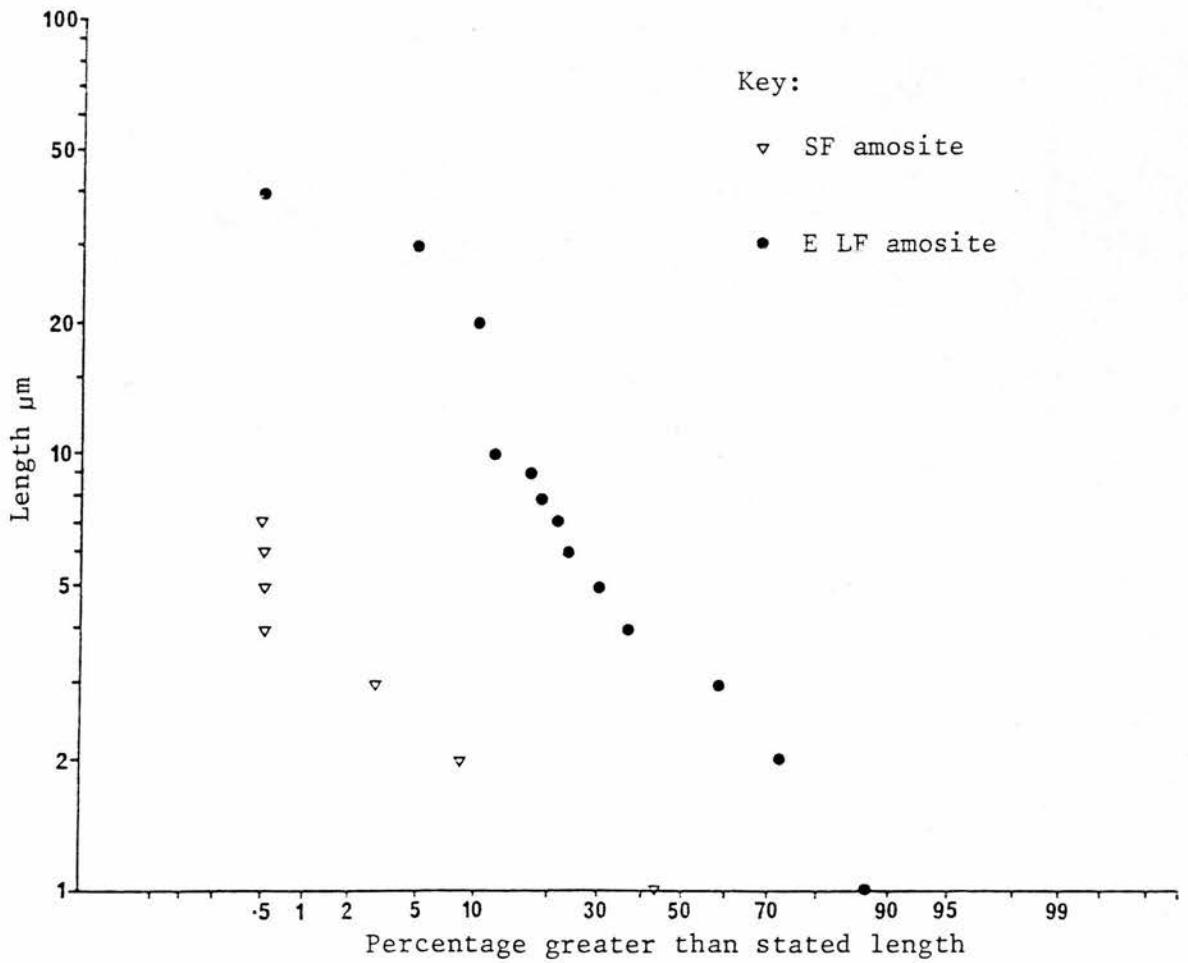

Figure 2.21 Fibre Length Distributions for SF Amosite and E LF Amosite Using 0.2 µm Pore Filter.

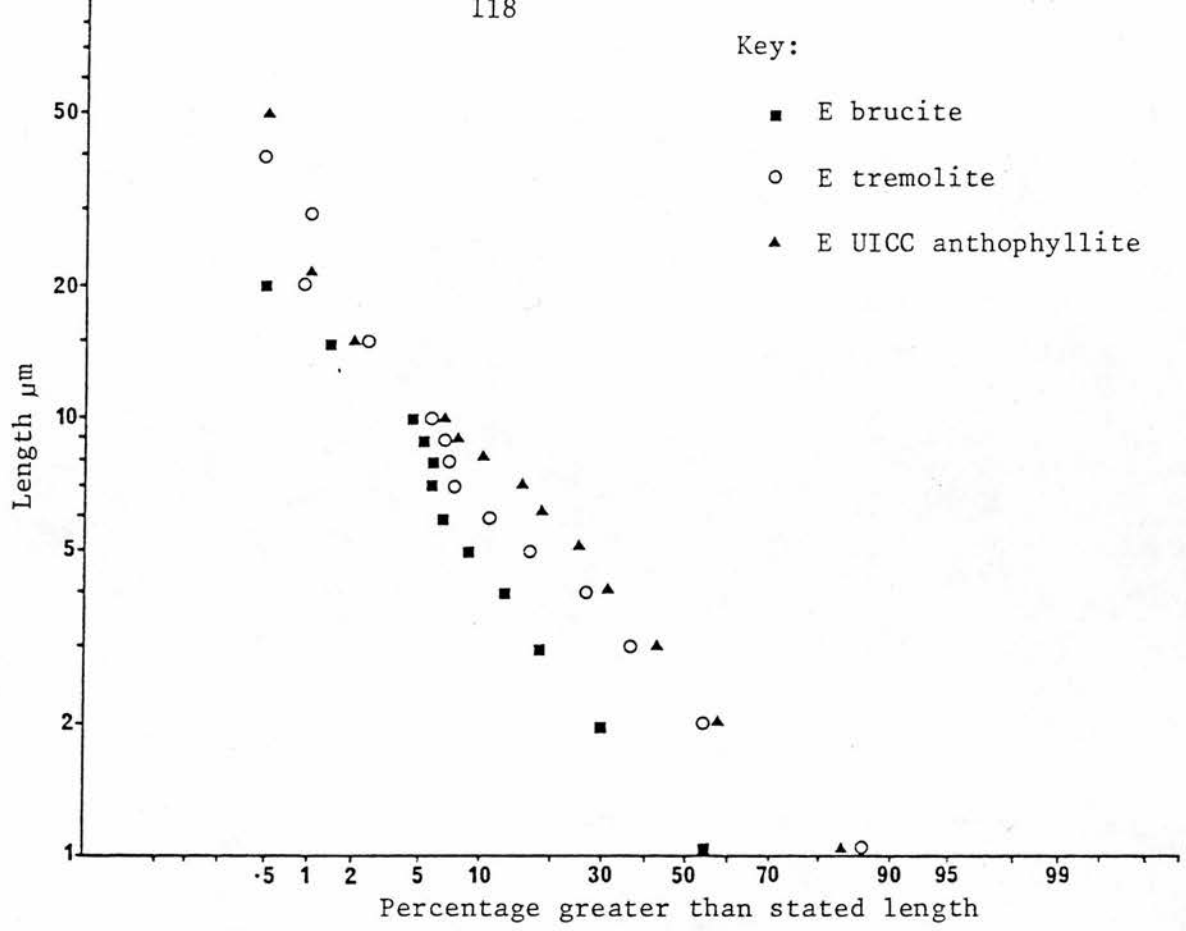

Figure 2.22 Fibre Length Distributions for E Tremolite, E UICC Anthophyllite and E Brucite Using 0.2 µm Pore Filter.

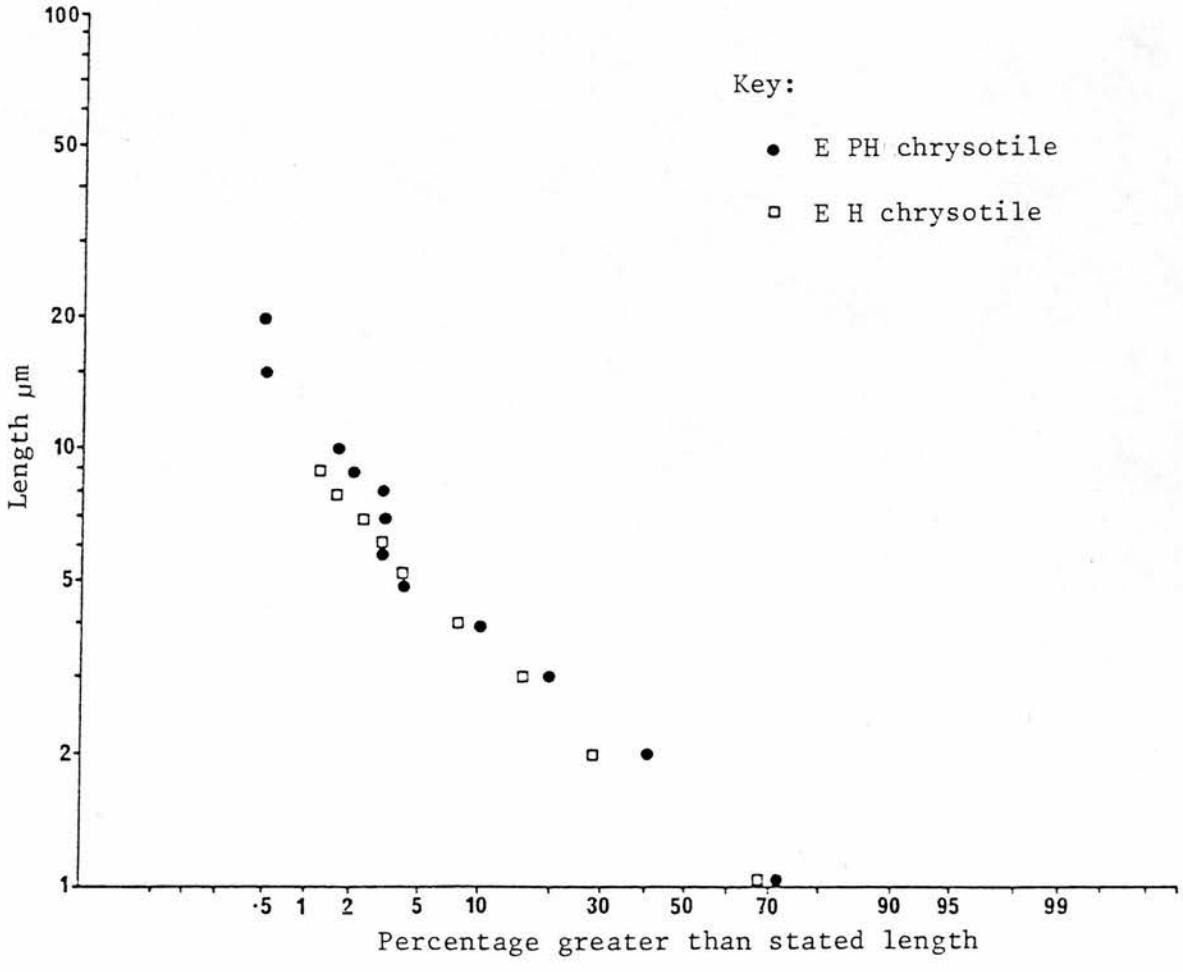

Figure 2.23 Fibre Length Distributions for E PH Chrysotile and E H Chrysotile Using 0.2 µm Pore Filter.

In general, it can be seen that the use of the 0.2 μm pore Nuclepore filter has proved successful in significantly increasing ($p<0.01$) the number of fibres of less than 1 μm in length and less than 0.2 μm in diameter which may be observed using the SEM.

2.3.3.4 Fibre Number Estimations.

The number of fibres observed for each asbestos sample is shown in Table 2.15, and is expressed in terms of the number of fibres per 10^{-10} g. E ceramic, E brucite and E UICC anthophyllite were shown to possess a comparatively small number of fibres compared to the other fibrous samples, whereas the SF amosite, SFA, UICC and E UICC chrysotile contained a relatively high number of fibres. Elutriation of the UICC samples resulted in a significant increase in the number of amosite fibres ($p<0.001$), a decrease in the number of chrysotile fibres ($p<0.01$), but no alteration in the number of crocidolite fibres. Heating the parent chrysotile material did not appear to significantly alter the fibre number content of the sample.

The data concerning the fibre number and fibre length distributions for each sample have been used to calculate the actual number of fibres available that are greater than the stated length for each fibrous sample. These are shown in Tables 2.16 to 2.18, and will be used for comparison with the cytotoxicity data discussed in Chapter 4.

Table 2.15 Fibre Number in 10^{-10} g for Each Asbestos Sample.

Asbestos type	Fibre no in 10^{-10} g ± SD
UICC crocidolite	112 ± 10
UICC amosite	77 ± 6
UICC chrysotile A	185 ± 18
E UICC crocidolite	109 ± 10
E UICC amosite	112 ± 14
E UICC chrysotile A	144 ± 20
SF amosite	169 ± 15
E LF amosite	15 ± 0.4
E brucite	19 ± 1
E tremolite	44 ± 2.7
E UICC anthophyllite	22 ± 1.6
SFA chrysotile	152 ± 14
E ceramic	7.4 ± 0.5
E F amosite	58 ± 3.6
E F chrysotile	59 ± 3.7
E PH chrysotile	93 ± 7
E H chrysotile	100 ± 6.2

Table 2.16 Number of Fibres Greater Than Stated Length for Each Asbestos Type in 10^{-10} g.

Fibre length μm	UICC crocidolite	UICC amosite	UICC chrysotile	E UICC crocidolite	E UICC amosite	E UICC chrysotile
0	112	77	185	109	112	144
1	75.2	60.1	141.8	72	86.2	102.9
2	28.7	27.4	80.1	26.8	41.4	48.2
3	14.1	18.5	48.3	10.4	23.5	24.4
4	7.9	11.6	26.1	7.1	14	10.7
5	4.5	7.4	18.6	4.4	8.4	7.1
6	3.4	5.9	12.1	2.8	4.5	5.7
7	2.3	3.2	10.2	1.7	2.3	4.3
8	1.7	2.4	7.4	0.6	2.3	4.3
9	1.1	1.2	5.5	0.6	0.6	2.1
10	-	1.2	4.6	0.6	0.6	2.1
15	-	-	0.9	-	-	1.4
20	-	-	0.9	-	-	0.7
30	-	-	0.9	-	-	0.7
40	-	-	0.9	-	-	0.7
50	-	-	0.9	-	-	0.7
100	-	-	-	-	-	0.7

Table 2.17 Number of Fibres Greater Than Stated Length for Each Asbestos Type in 10^{-10} g (Continued).

Fibre Length μm	SF amosite	E LF amosite	E brucite	E tremolite	E UICC anthophyllite
0	169	15.3	19.2	43.9	21.9
1	74.4	11.8	10.9	38.2	18.3
2	14.4	9.7	5.9	24.6	12.4
3	5.1	7.6	3.6	16.0	9.3
4	0.9	5.8	2.6	11.8	6.7
5	0.9	4.9	1.7	7.6	5.5
6	0.9	3.8	1.4	4.7	4.1
7	0.9	3.5	1.2	3.2	3.3
8	-	3.2	1.2	3.0	2.2
9	-	3.0	1.0	2.8	1.6
10	-	2.7	0.9	2.4	1.4
15	-	1.9	0.3	1.1	0.4
20	-	1.6	0.1	0.4	0.2
30	-	0.8	-	0.4	0.1
40	-	0.2	-	0.2	0.1
50	-	-	-	-	0.1
100	-	-	-	-	-

Table 2.18 Number of Fibres Greater Than Stated Length for Each Asbestos Type in 10^{-10} g (Continued).

Fibre Length μm	SFA chrysotile	E ceramic	E F amosite	E F chrysotile	E PH chrysotile	E H chrysotile
0	152	7.6	58	59.1	93.6	100
1	116.3	3.6	43.8	41.4	67.6	66.5
2	61.6	2.0	25.8	17.2	37.8	29
3	33.5	1.7	15.4	11.6	18.7	15
4	22.1	1.3	9.9	6.3	10.3	8
5	13.0	1.0	8.2	4.8	4.2	4
6	8.4	0.8	5.9	3.3	3.3	3
7	7.6	0.8	3.9	2.1	3.3	2.5
8	5.3	0.7	3.0	1.8	3.3	1.5
9	3.8	0.6	3.0	1.8	2.4	1
10	2.3	0.5	1.5	1.5	1.9	-
15	-	0.4	0.6	1.2	1.0	-
20	-	0.4	0.3	0.6	0.5	-
30	-	0.2	0.3	0.3	-	-
40	-	0.1	0.3	0.3	-	-
50	-	-	0.3	-	-	-
100	-	-	-	-	-	-

2.4 DISCUSSION.

The possible role played by the dimensions of the fibrous samples in the initiation of a range of diseases in man has been recognised for a number of years; in particular it would appear that the longer fibres may prove more pathogenic than the shorter ones (reviewed by Harington *et al*, 1975). Evidence to support this hypothesis has been provided by data from *in vivo* and *in vitro* studies (Harington *et al*, 1975), and more recently, some attempts at correlating the degree of activity of a dust with the number of fibres of a particular length threshold have been made. *In vivo* studies by Stanton *et al* (1977) showed a close correlation between the carcinogenic potential and also the number of fibres of greater than 8 μm in length and less than 1.5 μm diameter present in a fibrous sample. *In vitro* studies have shown a close correlation between the number of fibres greater than 6.5 μm in length and cytotoxic activity towards V79-4 and A549 cells (Brown *et al*, 1978) and greater than 10 μm in length for activity towards mouse peritoneal macrophages (Chamberlain *et al*, 1979). The data available concerning the occurrence of a fibre length threshold above which a fibrous sample may become active towards cultured cells, in particular macrophages, is limited, and there is need for an increase in the number of studies examining this problem. In order to assess the relationship between fibre length and *in vitro* cytotoxicity, it is necessary to establish an accurate and reproducible asssay system for the assessment of the fibre dimensions of dust samples, and the establishment of such an assay system proved to be one of the major aims of the present study.

2.4.1 The Validation of the SEM System for Assessment of Fibre Dimensions.

A number of methods are available which may be used for the assessment of fibre dimensions, and these comprise light microscopy, SEM and TEM techniques (Chatfield, 1979). Unfortunately the low resolution of the light microscope (Le Guen *et al*, 1980; A Wright personal observation) precluded its use in this study, as it was essential that the maximum number of fibres in each sample be examined. The choice of technique was therefore reduced to those utilising either the SEM or TEM. For the examination of the smaller and finer

fibres in the samples the TEM would be superior to the SEM (Chatfield, 1979), but it has the disadvantage of restriction of observation of a proportion of the filter area due to the presence of grid bars, and also the possibility of inaccurate assessment of those fibres in contact with a grid bar. It has been reported that the TEM "grid bar problem" may be overcome by alignment of fibres in an electromagnetic field prior to filtration, and the use of parallel bar EM grids to support the membrane filter, the grid bars being placed in the same direction as the electromagnetic field (Brown et al, 1978). Certainly, this system would facilitate the measurement of those longer fibres that lie parallel to the direction of the field (p-fibres) (Timbrell, 1975), but the problem of measurement of the long fibres which lie at right angles to the magnetic field (n-fibres) and that may fall across a grid bar, would still occur. The procedure for SEM observation does not necessitate as many preparatory steps as the TEM procedure (Section 2.12), thereby reducing the number of stages at which fibres may be lost or damaged, and this is a property that is worth some consideration; in addition a reduction in the number of preparatory steps would result in a reduction in the overall period of time involved in sample preparation. The SEM technique for fibre assessment would appear the most advantageous at this time, although it must be borne in mind that the very fine fibres of less than 0.1 μm diameter may not be readily observed (Middleton, 1982).

The technique used in this study for making permanent preparations of fibres involved very little sample manipulation, and it could therefore be assumed that neither fibre loss or breakage would occur; unfortunately it was not possible to check this. Preliminary studies using 0.4 μm pore filters demonstrated that the preparatory and counting techniques were reproducible (Table 2.4i) and ii)), although very few fibres below either 1 μm in length or 0.2 μm in diameter were observed (Tables 2.5 and 2.6), and it must therefore be surmised that some fibre loss had occurred. This assumption was confirmed upon the use of 0.2 μm pore filters, as the number of short and fine fibres observed increased considerably. This result may also have been partly due to an increased resolution of the IOM's SEM, which occurred at the same time as the introduction of the 0.2 μm pore filters.

An examination of the filtrate and also the backing filter following 0.2 μm pore filtration confirmed a lack of fibre loss due to the filtration procedure, although it may not have been possible to observe the extremely fine asbestos fibres on the SEM. Further evidence for the reproducibility of the preparation technique was provided upon examination of the UICC amosite replicates (Table 2.8), and again this also confirmed the reproducibility of the author's counting technique. An occasional replicate did occur within the UICC amosite samples, which proved statistically different from a proportion of the remaining replicates, and this emphasises the importance of preparing at least two replicates for each fibrous sample in order that any inaccuracy due to the preparation or counting procedure be identified. The same rationale can also be applied to the field counts; the amosite field count assessments all proved reproducible (Table 2.10) but it is still important to examine duplicate filters to check both the counting and preparation procedures. For routine analysis of fibre number, fields counts and hence fibre number estimates were obtained from five areas of each filter, again, this procedure is important to ensure that an even distribution of fibres across the filter has been maintained. Unfortunately, there is very little published data available concerning the reproducibility of these types of preparation techniques, and thus a critical comparison of the author's fibre preparation procedure with those of other research workers is not possible.

It was necessary for the purposes of this study that the fibre preparation procedure should incorporate an adequate means of fibre dispersal. This procedure should therefore result in a considerable reduction in fibre aggregates, and in particular encourage the smaller fibres to detach from the larger ones. This would allow an optimal characterisation of fibre length distribution on the SEM , and also permit the cells in the cytotoxicity study an opportunity to phagocytose individual fibres rather than clumps. Ultrasonication was used for this purpose, but was found to induce an alteration in both fibre length and diameter distribution (Table 2.7) for the UICC samples. Chatfield et al (1978) and

Spurny et al (1980b) used ultrasound at a frequency of 20 and 50 KHz repsectively to disperse their samples, and found small alterations in the dimensions of their fibre samples, which were dependent on the length of exposure as well as the power and frequency of the ultrasonication procedure. Spurny et al (1980b) concluded that the alterations in fibre concentration and size were smaller than the statistical errors resulting from fibre measurement, and, more importantly, he showed that low levels of ultrasonication did not alter the crystalline structure of asbestos (Spurny et al, 1980b). Alternative methods of fibre dispersal would involve shaking, squirting the solution through a pipette or syringe, or alternatively sieving; all of these techniques would prove more difficult to standardise than the ultrasonication procedure. It can therefore be concluded that whilst ultrasonication is the most convenient means available for fibre dispersal, some alteration in fibre dimensions may occur, but this may not be too important if the dimensions of each sample can be accurately characterised following exposure to ultrasonication. It was unfortunate that the ultrasonication procedure did not encourage an adequate dispersal of the E WDC sample, thereby limiting the possibility of assessment of the fibre dimensions. This result would suggest that the WDC process described by Heron and Huggett (1971) is indeed successful in producing strands of fibres that are firmly bonded together. It is possible that the application of a more rigorous dispersal procedure would have resulted in a successful separation of the fibres, but under such circumstances the integrity of the fibrillar structure may also have been altered. There is no published data available which describes a method for preparation of WDC for SEM or TEM observation, and thus no further examinations of the morphology of the WDC samples were carried out in this study.

The data from the UICC amosite replicates (Tables 2.8 to 2.10) showed that the fibre preparation technique ie. ultrasonication dilution and filtration, and also the author's fibre counting technique were reproducible. It is now accepted that some differences may be obtained upon the comparison of results from fibre counters from

different laboratories, and this has been shown to be true for observations using the light microscope (Beckett and Attfield, 1974). An inter-laboratory comparison using TEM techniques (Gaudichet et al 1980), has shown, however, that comparable results may be obtained. More recently a study has been carried out at the IOM to ascertain the degree of inter-observer variation during the use of the SEM (Cherrie et al, in press). A considerable variation in both fibre length distribution and fibre number counts was shown; the variation appeared to depend upon the enthusiasm of the counter involved and could be stated as "the harder one looks, the more fibres one finds". The author (AW) was involved in this study, and her counting results fell within the range of those fibre counters who proved the most observant. A separate comparison between the author and a "reliable" fibre counter from the IOM showed that their fibre assessments were very similar (Table 2.11). It can reasonably be assumed, therefore, that the dimensions and number of fibres recorded by the author were an adequate representation of those actually present on the filter.

2.4.2 The Fibre Dimensions and Fibre Number Data.

The data concerning the lengths and diameters of the various asbestos samples has been expressed in this chapter in terms of the percentage of fibres greater than the stated length for each population of fibres. This form of presentation for the fibre data is consistent with previous reports from the IOM (Davis et al 1978, Wright et al 1980). There are a number of alternative ways of describing fibre length data, including percentage less than stated length (Brown et al, 1978), log fibre length versus corresponding frequency (Siegrist and Wylie, 1980), or as the absolute percentage of fibres in each length category (Langer et al, 1974; Coffin and Palekar, 1978; Rendall 1980). The differences in types of presentation may create some difficulty to the reader during the comparison of results from different reports; all of the data used for comparison in this chapter has therefore been reconverted and expressed in terms of the percentage of fibres greater than stated length.

The examination of the fibre length and diameter distributions in this study involved the use of 0.4 and 0.2 μm pore membrane filters. In general, it was found that a proportion of the smaller fibres were not evident upon examination of the 0.4 μm pore filters, and the results were therefore not representative of the total population. The results from the 0.2 μm pore filters only will be discussed here. The fibrous samples examined in this study had been subjected to an "elutriation" process to ensure the exclusion of non-respirable fibres from each sample. A comparison between the elutriated and non-elutriated versions of UICC crocidolite, amosite and chrysotile showed that no significant alteration in fibre length distribution had occurred by virtue of the elutriation process (Table 2.12). The UICC samples were prepared by Timbrell (1970) in a manner such that all of the fibres would be of a respirable size range and thus the lack of effect during elutriation would be expected. Some alteration in fibre number was observed however (Table 2.15), an increase in fibre number for E UICC amosite, and a reduction for E UICC chrysotile. The amosite result could be due to the loss of a low percentage (1%) of those large and thick fibres with a high mass value; the chrysotile result is inexplicable except in terms of loss of a proportion of finer fibres via the filter pores.

The data available in the literature concerning fibre length assessment of those fibrous samples examined in this study is sparse, and is mainly concerned with estimations of the UICC samples using the TEM. Timbrell (1970) characterised UICC amosite, anthophyllite, crocidolite and chrysotile A using TEM techniques (Table 2.19). The length distributions for both crocidolite and anthophyllite were similar to those reported for this study; although a greater number of fibres of less than 5 μm in length were reported by Timbrell for both amosite and chrysotile (30% rather than 9% for amosite, and 20% rather than 6% for chrysotile). Langer et al (1974), again using the TEM, showed that both amosite and anthophyllite had a greater number of fibres of length greater than 5 μm when compared to the results obtained in this study (16% and 39% respectively) (Figure 2.20), and crocidolite proved considerably shorter (37% greater than 1 μm rather than 67%). Brown et al (1978) expressed their TEM assessments of crocidolite, anthophyllite and amosite graphically,

Table 2.19 Fibre Length Distributions for TEM Preparations of UICC Samples.

After Timbrell (1970).

Asbestos type	Percentage longer than stated length μm					
	1	2	5	10	15	20
Amosite	70.5	54.6	31.1	15.1	7.3	4.9
Anthophyllite	66.7	50.5	28.6	11.5	5.8	3.2
Crocidolite	60.6	32.0	8.5	2.8	0.9	0.5
Chrysotile A	81.7	61.1	25.4	7.8	2.7	0.9

Table 2.20 Fibre Length Distributions for TEM Preparations of UICC Samples.

After Langer et al (1974).

Asbestos type	Percentage longer than stated length μm				
	1	2	3	4	5
Crocidolite	37	15	7	4	2
Amosite	77	49	27	20	16
Anthophyllite	86	66	53	45	39

Table 2.21 Fibre Length Distributions for TEM Preparations of UICC Samples.

After Rendall (1980).

Asbestos type	Percentage longer than stated length μm		
	1	2	5
Amosite	52.1	16.1	1.8
Crocidolite	54.9	12.8	2.2

and more encouragingly found similar results to those reported in this chapter, particularly with respect to the number of shorter fibres, although a greater number of fibres of longer than 6 μm for amosite (30% rather than 7%) was noted. In contrast, Coffin and Palekar (1978) showed that only 13% of their UICC amosite fibres were greater than 5 μm, and Rendall (1980) (Table 2.21) reported that approximately 2% only of both amosite and crocidolite fibres were greater than 5 μm. In general, the reports are confusing; there is little agreement concerning the fibre length distributions, and this can probably be ascribed to differences in fibre preparation and dispersal, possible differences between observers, and also the use of different counting rules. The differences between the findings of the author and those of previous reports may also be due to the utilisation of the SEM in this study, compared to the TEM in other reports. Data concerning fibre dimensions is consistent with the suggestion that it is possible to find more fine fibres using the TEM rather than the SEM (Middleton, 1982), as the fibre diameter distributions reported by Langer et al (1974) and Rendall (1980) for UICC amosite, anthophyllite, crocidolite and chrysotile A, show a greater proportion of finer fibres than reported for this study (Table 2.22 and 2.23). The discrepancy between the SEM and TEM counts occurs for those fibres of 0.6 μm diameter or less; this may be due to "background noise" on the SEM viewing screen or even effects due to the "charging up" of fibres, which would effectively obscure the sharp image of the finer fibres, thereby minimising their accurate assessment.

A few groups of researchers have attempted to estimate the number of fibres present in their asbestos samples (Monchaux et al, 1981; Brown et al, 1978) and their results are shown in Table 2.24, together with results from this study for comparison. It is encouraging to note that the number estimates carried out by Brown et al (1978) are in the same order of magnitude as those found in this study, despite the use of different techniques. Monchaux et al (1981) however, recorded a greater number of fibres, especially for UICC chrysotile where a ten-fold increase was observed. Monchaux et al, (1981) prepared their samples for TEM observation by using ultrasonication, seiving and also by flushing their samples through a 21 gauge needle.

Table 2.22 Diameter Distributions for TEM Preparations of UICC Samples.

After Langer et al (1974).

Asbestos type	Percentage thicker than stated diameter μm 0.1	0.2	0.3	0.4	0.5
Amosite	100	44	32	22	9
Anthophyllite	100	69	54	42	33
Crocidolite	100	33	18	13	8

Table 2.23 Diameter Distributions for TEM Preparations of UICC Samples.

After Rendall (1980).

Asbestos type	Percentage thicker than stated diameter μm 0.1	0.2	0.3	0.4	0.5	0.6	0.7
Amosite	100	56.7	29.8	13.0	6.8	3.5	1.9
Anthophyllite	100	69.5	51.9	34.7	22.3	16.2	10.6
Crocidolite	100	45.7	19.3	9.1	4.2	1.5	0.4
Chrysotile A	100	28.3	8.1	3.3	0.9	0.3	0.2

Table 2.24 Comparison of Fibre Number Counts From Different Sources.

Asbestos sample	Number of fibres per 10^{-10} g Thesis	Brown et al (1978)	Monchaux et al (1981)
UICC amosite	77	46	-
UICC crocidolite	112	160	304
UICC anthophyllite	22	15	-
UICC chrysotile A	185	-	1150

This type of preparation technique is rather traumatic, and it is therefore not surprising that the fibre number estimates should be so great. The differences observed between the fibre number counts obtained for this study and those reported by Brown et al (1978) can probably be ascribed to the different preparation techniques and types of microscopical examination used.

There would appear to be few reports available in the literature concerning the fibre length and diameter distributions of the remaining fibrous samples examined this study. It is worthy of note, however, that the E factory samples of chrysotile and amosite possessed an unexpectedly low number of fibres (Table 2.15), and both of these samples were found to be contaminated with particulate material from the factory environment, E F amosite having approximately 10% of its weight in particulate matter, and E F chrysotile 40% (Bolton et al, 1982a). It must also be considered that, in addition to contamination with particulate material, adsorbed carcinogens and other biologically active materials from the factory environment may have been present, and some caution must be exerted when interpreting results from experimental data concerning the effects of these dusts in vitro and in vivo.

It is unfortunate that there are very few reports available that have systematically compared results from both SEM and TEM using similar preparation techniques, and the situation illustrates that there is a strong requirement for this type of study to be carried out. In particular the majority of those fibre estimates involving an examination of filtered fibrous material would appear to have been carried out on the TEM, and it is therefore not possible to undertake a comparison of the SEM results obtained in this study with SEM results from other studies. Comparisons of the data obtained from this study with TEM studies have proven slightly confusing, whilst it would appear from the data reported by Timbrell (1970) and Brown et al (1978) that a similar proportion of short fibres may be detected regardless of whether the TEM or SEM is used, a greater proportion of longer fibres were detected using the TEM; Rendall (1980) however, found very few long fibres when using the TEM. Brown et al (1978) counted 1000 fibres during their studies, and it

is possible that counting only 200 fibres (as used in this study) was not adequate for the inclusion of a representative number of longer fibres. Alternatively, the counting rules used in the TEM studies may have been different to those employed in this study, which were designed to avoid any bias towards the "over-assessment" of longer fibres; as none of the publications reported in this chapter have described their counting rules, it is not possible to discuss them. The reports by Langer et al (1974) and Rendall (1980) confirm the suggestion by Middleton (1982) that it is possible to observe a greater number of finer fibres using the TEM, and this highlights a major disadvantage of using the particular SEM involved in this study. This situation could possibly be improved by assessing SEM photographic images of fibres (Middleton, 1982). The fibre number counts of Brown et al (1978) were found to be in the same order of magnitude as the counts obtained in this study, and this encouraging feature would suggest that it is worthwhile pursuing the utilisation of the SEM as a convenient method for fibre assessment.

One of the major findings to evolve from this study is the urgent need for further studies to be undertaken comprising a comparison and standardisation of fibre preparation and counting techniques. In addition, it is necessary that inter-personal and inter-laboratory assessments of fibre counting procedures be examined to ascertain the variability involved. A number of studies of this nature are already underway, and hopefullly more will be undertaken soon, in order that this complicated situation be clarified in the near future.

2.5 CONCLUSIONS.

This study comprised the establishment of a technique involving the use of the SEM for assessment of fibre dimensions and fibre number. The preparation techniques and the fibre counting procedure were shown to be highly reproducible. The SEM technique was used to establish the fibre length and fibre diameter distributions as well as the fibre number content for 17 different asbestos samples, although it did not prove useful for assessing a number of commercially prepared samples of wet-dispersed chrysotile. A comparison between assessments

using the SEM, and reports in the literature concerning assessments using the TEM, would suggest that the TEM technique is superior because of its higher resolving powers; the use of an improved, modern SEM may however challenge this situation.

CHAPTER 3 THE HAEMOLYTIC ABILITY OF ASBESTOS FIBRES.

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CHAPTER 3 THE HAEMOLYTIC ABILITY OF ASBESTOS FIBRES.

3.1 INTRODUCTION.

The recognition that the majority of asbestos types and also silica particles can induce fibrosis in man and animals, has led to the search for an *in vitro* model which may be used for the prediction of the *in vivo* pathogenicity of dust samples, and also for the detailed examination of dust/cell membrane interactions (reviewed by Harington *et al*, 1975). Since the 1950s a number of research groups have held the opinion that the erythrocyte would adequately fulfil this role; the use of this cell model has already been described in detail in Section 1.13.4 of this thesis, and the more important findings only will be reiterated in this section.

The particulate form of silica is particularly haemolytic in nature (Dognon and Simonot, 1951; Macnab and Harington, 1967), and a number of mechanisms have been suggested by which silica may disrupt the erythrocyte membrane. The surface of the silica particle becomes negatively charged in physiological solutions by virtue of the formation of silanol groups (Nash *et al* , 1966; Nolan *et al*, 1981), and it has been suggested that these groups may interact with membrane lipids (Allison, 1971; Weismann and Rita, 1972) possibly by inducing lipid peroxidation (Gabor and Anca, 1974), or alternatively with membrane proteins (Summerton *et al*, 1977). More recently Depasse (1977, 1980) has shown that erythrocyte lysis may occur following the interaction of silica with the trimethylammonium group of the membrane lecithin molecule. With regard to the haemolytic potential of asbestos fibres, it has been shown that the serpentines are more haemolytic than the amphiboles (Vigliani, 1968; Macnab and Harington, 1967; Light and Wei, 1980). These findings have been ascribed to the magnesium content of the fibres (Harington *et al*, 1971b) by virtue of the ability of the magnesium to react with the sialic acid residues of the erythrocyte membrane (Harington *et al*, 1971b); Depasse, 1982). Alternatively Light and Wei (1977a, b, 1980) have demonstrated that there is a role for the surface properties of an asbestos fibre, as manifest by the zeta potential, in determining its haemolytic capacity. In the case of both silica and asbestos it would appear

that the mechanism of interaction with the erythrocyte membrane has not yet been determined, and there is still room for further research into this problem. Hefner and Gehring (1975) demonstrated that there is a relationship between the haemolytic capacity of particles and their ability to induce fibrosis, and they suggested that the interaction of a particle with the erythrocyte membrane may reflect its interaction with macrophage lysosomal membranes. It would appear therefore, that an examination of the haemolysis assay may prove fruitful, as results from this system may aid in the interpretation of data from cytotoxicity experiments.

3.1.1 Aims and Objectives.

The aims of this study were:

i) to establish the haemolytic capacity of the available fibrous samples.

ii) to assess the usefulness of the haemolysis assay as a model for the examination of fibre/membrane interactions.

3.2 MATERIALS AND METHODS.

3.2.1 The Types of Fibre Samples Examined.

The following fibrous samples were examined in this study:

E ceramic fibre

UICC crocidolite

UICC amosite

E UICC amosite

E F amosite

UICC chrysotile A

E PH chrysotile

E H chrysotile

SFA chrysotile

E F chrysotile

E WDC

DQ_{12} - a positive control, selected for its known haemolytic activity towards erythrocytes (Robock, 1973).

TiO_2 (rutile form) - a negative control, selected for its known low haemolytic activity (Ottery and Gormley, 1978).

A number of the fibrous samples previously described in Section 2.2.2 of this thesis were not examined in this study because they were not available at the appropriate time.

3.2.2 Preparation of Fibre Samples.

A sample of each dust (10.0 mg) was suspended in veronal buffered saline (VBS) pH 7.2 (Harington et al, 1971b) so that a final dust concentration of 2 mg/ml was achieved. The suspended samples were dispersed by ultrasonication for 2 mins at 100 KHz as described previously (Section 2.2.4.2).

3.2.3 Preparation of Erythrocytes and Standardisation of Erythrocyte Suspension.

Freshly harvested citrated sheep erythrocytes were obtained from Gibco-Biocult Laboratories, and were used within one week of receipt.

The erythrocytes were suspended in VBS (Harington et al, 1971b) and centrifuged (MSE super-minor) at 500 g for 10 mins; this washing procedure was repeated 3 times to ensure the removal of serum proteins.

In order to ensure that the erythrocyte concentration was standardised for each experiment the following procedure was employed:

1) 2 mls of packed erythrocytes were suspended in 100 mls of VBS.
2) 1 ml of erythrocyte suspension was totally lysed with 1 ml of 0.1% Triton-X (Koch-Light) in distilled water.
3) The optical density (OD) at 541 nm for haemoglobin release for the totally lysed sample was assessed using a Pye Unicam SP 30 spectrophotometer.
4) The concentration of the erythrocyte suspension was adjusted using either packed erythrocytes or VBS, until an OD of 1.500 was obtained for the totally lysed sample.

The standardised erythrocyte suspension was employed for the remainder of the experiment.

3.2.4 Haemolysis Assay.

The haemolytic ability of the dust samples was established as follows:

1) 1ml of sheep erythrocyte suspension was added to 1 ml of dust suspension in an RT 30 tube (Sterilin) to give a final dust concentration of 1 mg/ml. Duplicate tubes were prepared for each dust sample.
2) Duplicate tubes containing 1 ml of erythrocytes and 1 ml of VBS (Blank Control) were prepared so that the haemoglobin release due to spontaneous lysis of erythrocytes could be assessed.
3) The tubes were incubated at 37 °C and mixed by careful inversion every 5 mins.
4) After 30 mins the tubes were centrifuged at 500 g for 10 mins and the supernatant removed.
5) For each tube, the OD of the supernatant at 541 nm was measured.

3.2.5 Presentation of Data and Statistical Analysis.

The percentage haemolysis for each tube was calculated as follows:

$$\% \text{ haemolysis} = \frac{\text{OD for test tube - OD for "blank" control}}{\text{OD for totally lysed (Triton-X) sample}}$$

The experiment was repeated on 2 occasions, thereby resulting in a total of 4 percentage haemolysis values for each dust. The final percentage haemolysis value is expressed as a mean value together with the standard deviation.

Statistically significant differences between the haemolysis values for the different dust samples were assessed using the Student T Test (Bailey, 1974).

3.3 RESULTS.

The haemolytic ability for each dust sample, which has been reported previously (Gormley et al, 1980; Wright et al, 1980), is shown in Table 3.1, and is expressed in terms of the percentage haemolysis. It can be seen that the serpentine samples were considerably more haemolytic than the amphiboles ($p<0.01$ for UICC amosite and SFA chrysotile). The amphiboles together with E ceramic fibre and the TiO_2 control showed a lower degree of haemolytic activity; whereas the DQ_{12} proved to be the most haemolytic dust sample ($p<0.05$ for E UICC chrysotile and DQ_{12}), and E UICC chrysotile possessed a greater haemolytic ability than all of the other asbestos sample examined ($p<0.02$ for UICC chrysotile and E UICC chrysotile). E H chrysotile exhibited a significantly lower degree of haemolysis than the E PH chrysotile sample ($p<0.01$ for E PH chrysotile and E H chrysotile).

Both the SFA chrysotile and E WDC samples had haemolytic capacities that were not significantly different from the value for E H chrysotile ($p>0.05$ for SFA chrysotile, E WDC and E H chrysotile). The E WDC sample proved particularly difficult to disperse in solution due to the formation of fibre "mats", and these tended to reduce the efficient maintenance of fibre/erythrocyte interactions during the 30 mins incubation period.

Table 3.1 The Haemolytic Activity of Asbestos Fibres.

Fibre Type	% Haemolysis
TiO_2	0.1 ± 0.1
E ceramic	0.7 ± 0.7
UICC crocidolite	0.0
E UICC amosite	0.0
E F amosite	0.0
UICC amosite	2.7 ± 0.7
E H chrysotile	34 ± 2.0
SFA chrysotile	34 ± 4.7
E F chrysotile	44 ± 1.3
E WDC	48 ± 11.4
E PH chrysotile	67 ± 0.7
UICC chrysotile	72 ± 1.3
E UICC chrysotile	85 ± 1.7
DQ_{12}	94 ± 1.0

Results are a mean of 4 readings, resulting from 2 duplicates from each of 2 separate experiments.

3.4 DISCUSSION.

The continued study of the erythrocyte haemolysis assay was assured for a number of years, because of its possible toxicological usefulness as a predictor of *in vivo* fibrogenicity of mineral dusts (Hefner and Gehring, 1975). A close correlation was observed between haemolysis and cytotoxicity assays (Koshi *et al*, 1968) which would also suggest that the interaction of a given dust sample with the erythrocyte membrane may possibly reflect its interaction with the macrophage membrane (Harington *et al*, 1975). In this study erythrocytes have been exposed to a selection of dust samples, so that a comparison could be made between their haemolytic abilities and their cytotoxic activities towards macrophages.

The haemolytic abilities of the various dust samples are shown in Table 3.1. The rutile form of TiO_2, in agreement with previously reported data (Ottery and Gormley, 1978; Zitting and Skytta, 1979), showed a low degree of haemolysis, whereas DQ_{12}, in agreement with Stalder and Stöber (1965) and Robock (1973) proved to be very haemolytic. The observation that the amphiboles were less haemolytic than the chrysotile samples is also in agreement with other reports (Harington *et al*, 1971b; Light and Wei, 1980). The haemolytic capacity of of the fibre samples might be explained in terms of one or a combination of factors, and these factors involve either the fibre number, the surface properties and/or the ability of the sample to be dispersed adequately in suspension. The fibre number per unit weight is important, as this governs not only the concentration of test material available but also the surface area available to interact with the cell membrane. It has been suggested that the haemolytic capacity of a dust sample may be dependent upon the particle number per unit mass (Ottery and Gormley, 1978), this theory, however, is not wholly compatible with the results obtained in this study. Ceramic fibres, which proved non-haemolytic also possessed a very low fibre number (7.4 fibres/10^{-10} g, Table 2.15); whereas E UICC chrysotile proved more haemolytic than UICC chrysotile despite its apparent reduction in fibre number from 185 to 144 fibres/10^{-10} g. E F chrysotile and E F amosite possessed fibre number counts which were not significantly

different from each other, although the former sample proved far more haemolytic than the latter. SFA chrysotile, another sample with a relatively high fibre number (144 fibres/10^{-10} g), proved unexpectedly low in haemolytic activity (34%).

The "surface properties" of a particular fibre sample are possibly the most important parameter governing the haemolytic ability of the fibres. Light and Wei (1977a, b, 1980) explained the haemolytic ability in terms of the surface properties as manifest by the surface charge (zeta potential) of the fibre. The results obtained in this study agree with those of Light and Wei, in that the amphiboles, which possess a negative zeta potential, were non-haemolytic, whereas the chrysotiles, which possess a strong negative zeta potential, were markedly haemolytic. Another finding which illustrates the importance of the surface properties was that E H chrysotile (850 °C) showed a 50% reduction in haemolytic ability compared to the E PH chrysotile sample. This result agrees with data reported by Hayashi et al (1969) who showed that at temperatures of 800 °C and above, the haemolytic capacity of chrysotile was considerably reduced. This finding was ascribed to the breakdown of the bonds between the Si-O tetrahedra, thereby allowing the tetrahedra to rotate, become rearranged and eventually form the non-haemolytic forsterite structure (Hayashi et al, 1969). The low haemolytic ability of the SFA chrysotile sample may also be explained in terms of an alteration in surface properties of the fibres. Langer et al (1978) reported that milling of chrysotile serves to alter the structure of the brucite layer, thereby reducing the adsorptive and also the haemolytic capacity of the sample. The SFA chrysotile was prepared by milling, and it has been shown that the sample has a reduced ability to absorb albumin as compared to the parent sample (Morgan, 1974), thereby suggesting that the surface properties of SFA chrysotile have been dramatically altered.

Another factor governing the haemolytic capacity of a fibrous sample is its ability to disperse adequately in solution. A report by Sykes et al (1980) demonstrated that the haemolytic ability of chrysotile may vary according to the degree of "opening" (dispersal) of the fibre sample, an increased degree of opening resulting in

an increase in the area of fibre surface available to interact with the erythrocyte membrane. The lower haemolytic ability (48%) of the E WDC sample as compared to the UICC chrysotile samples can be explained in terms of the inability of the sample to open and disperse adequately in suspension, thereby reducing the number of fibre/cell interactions. In addition, the large standard deviations observed for the E WDC results (Figure 3.1) may be explained in terms of "fibre mats" hampering the reproducibility of the fibre/cell interactions. The design of the experiment did not allow the continual agitation of the fibre sample and erythrocytes during the test period, and possibly the incorporation of an agitating mechanism in the assay system would improve this situation, although it must be borne in mind that this type of system may result in an increased erythrocyte lysis because of the cells mechanically shearing against the particles.

David and Roth (1976) queried the use of the haemolytic assay as a predictor of fibrogenicity as the results are complex, dependent on a number of factors such as the particle number and surface properties of the sample, and the assay does not allow adequate facilities for the interpretation of results obtained from the examination of mixed dust samples. David and Roth concluded that the system could only be used as a model for the investigation of the interaction of dust particles with cell membranes. Later reports by Daniel and Le Bouffant (1980), Gormley et al (1980) and Wright et al (1980), however, showed a number of discrepancies when comparing the results from the haemolysis assay with data from systems employing macrophages; in addition, Richards et al (1980) noted a lack of correlation between the haemolysis assay and their in vivo results. This finding was supported to some extent by the report of Scott and Furcht (1976) who indicated that the erythrocyte plasma membrane is very different in structure from that of other cells; this would suggest that the future used of the haemolysis assay as a model for fibre/macrophage interactions would not be reasonable.

After consideration of the problems concerning the haemolysis assay that have been highlighted in this discussion, it was decided that, whilst the haemolysis data might prove useful for comparison

with the macrophage cytotoxicity data, it was not reasonable to assume that the results from the fibre/erythrocyte interaction would reflect or even attempt to explain the mechanism of fibre/macrophage interactions, or prove predictive with regard to in vivo pathogenicity. For these reasons, no further work was carried out using this system.

3.5 CONCLUSIONS.

The haemolytic abilities of a number of fibrous samples were examined in this study. It was concluded that the results from the haemolysis assay may not necessarily contribute towards explaining results obtained from macrophage cytotoxicity experiments.

CHAPTER 4 THE CYTOTOXICITY OF FIBROUS DUSTS TOWARDS P388D$_1$ CELLS - WITH REFERENCE TO FIBRE DIMENSIONS.

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CHAPTER 4 THE CYTOTOXICITY OF FIBROUS DUSTS TOWARDS P388D$_1$ CELLS - WITH REFERENCE TO FIBRE DIMENSIONS.

4.1 INTRODUCTION.

The alveolar macrophage plays a key role in defence of the lung, being endowed with the ability to ingest inhaled particulate and microbial organisms, to destroy inhaled biological pathogens and also tumour cells, and secrete a variety of products which enable the phagocyte to interact with the immunological system of the body (see review by Nathan *et al*, 1980). The recognition that one important function of the macrophage involved the phagocytosis and clearance of inhaled dust particles from the lung, prompted the initiation of a number of studies to examine the efficiency with which this particular cell carried out its task (see review by Harington *et al*, 1975), and these mainly comprised a direct examination of the effects of various mineral dusts on macrophages *in vitro*. The observation that the cytotoxic capacity of various forms of silica towards macrophages paralleled the ability of each form to stimulate collagen production in animals (Marks, 1957; Allison, 1971) led to an extensive examination of the usefulness of this *in vitro* cell model as an indicator of the *in vivo* fibrogenicity of a dust sample. The asbestos story proved more complex, however, as the *in vitro* effects of the various types of asbestos did not adequately reflect their *in vivo* fibrogenic capacities; it was demonstrated that chrysotile initiated a considerable loss of intracellular enzymes from macrophages whereas the amphiboles appeared relatively inert (Koshi *et al*, 1968; Allison, 1971), despite the capacity of all types to induce fibrosis *in vivo* (Wagner *et al*, 1974). Davies *et al* (1974a) later demonstrated that chrysotile asbestos had the ability to induce a selective release of lysosomal hydrolases by macrophages in the absence of cell death, and this finding led to the interesting concept that *in vivo* fibrosis may be induced not only as a result of macrophage death, but also leakage of enzymes from viable macrophages (Davies and Allison, 1976). These findings and extrapolations have contributed to ensuring the continued study of the effects of asbestos and silica on macrophages, with particular reference to their mechanism of interaction and their ability to induce enzyme release. These studies have been extensively reviewed by Harington *et al* (1975), Harington (1976) and

Miller (1978) and also Section 1.13.5 of this thesis, and thus only the more pertinent features will be described in the following section.

4.1.1 The Mechanism of Interaction of Silica and Asbestos With Macrophages.

Numerous studies have been carried out in an attempt to establish the mechanisms by which silica and asbestos interact with the macrophage membrane (Section 1.13.5.1 and 1.13.5.2 to 1.13.5.4); and whilst a number of these have indicated a variety of possible mechanisms of action, none have proven definitive. The cytotoxic nature of silica has been ascribed to a number of interactions involving i) the ability of silicic acid to form hydrogen bonds with lipid or protein components from the cell membrane or phagolysosomal membrane (Allison et al, 1966; Nash et al, 1966) ii) the ability of silica to activate the macrophage phospholipase A system and also inhibit membrane-bound acyl-transferase, thereby resulting in an accumulation of surface-active lysophosphatides (Munder et al, 1966, 1967; Munder and Lebert, 1977), alternatively iii) lipid peroxidation may be involved in silica-induced toxicity (Gabor et al, 1980). With respect to the cytotoxic action of asbestos, the situation displays a similar degree of complexity, and emphasis has been placed on establishing the mechanism employed by chrysotile which results in a release of intracellular enzymes. The ability of chrysotile to interact with the macrophage membrane has been ascribed to the surface properties (Light and Wei, 1980), and also the magnesium content (Miller and Harington, 1972; Kaw and Zaidi, 1975) by virtue of an interaction with either the plasma membrane glycolipids (Miller and Harington, 1972) or lipid-bound sialic acid groups (Depasse, 1982). Alternatively chrysotile may induce a release of phospholipase A from macrophages (Sirois et al, 1980), thereby resulting in an increase in lysolecithin (Miller and Harington, 1972) followed by membrane damage. However, none of these mechanisms have adequately clarified the situation with respect to the inactivity of the amphibole asbestos types towards macrophages in vitro, despite their apparent fibrogenic activity in vivo (Harington et al, 1975). A number of research groups have therefore turned to examining the role of the fibre length in determining the cytotoxic potential of a fibrous sample.

Early studies by Bey and Harington (1971) and Robock and Klosterkötter (1971) demonstrated that samples of asbestos enriched with smaller fibres were less active towards macrophages than material containing long fibres. Later studies by Davies (1980a,b), Davies et al (1980), Beck and Tilkes (1980) and Kaw et al (1982) (reviewed in Section 1.13.5.4) have provided further evidence that those dust samples which contain a greater proportion of longer fibres also possess a greater capacity to induce enzyme loss from cultured macrophages. Unfortunately only one study has attempted to correlate the degree of enzyme release from macrophages with a particular length threshold, and this study (Chamberlain et al, 1980) has shown a close correlation between the number of fibres greater than 10 μm in length and the degree of enzyme loss. Certainly it would appear that there may be a link between fibre length, enzyme release from macrophages in vitro and fibrogenicity in vivo, but it is evident that further work is required, in particular with reference to enzyme release from macrophages, to substantiate the hypothesis concerning the existence of a fibrogenically active fibre length threshold.

4.1.2 The Selection of the Type of Macrophage to be Used for Examination of Asbestos/Macrophage Interactions With Respect to Fibre Length.

The examination of macrophage/asbestos interaction is hampered to some extent by the problems involved in selecting a relevant macrophage population for study. It is now accepted that macrophage populations may be obtained in a variety of activational states, depending on the source of the population and the nature of any prior stimulating agents to which the population may have been exposed; the functional properties of each population may vary considerably depending upon the activational status (Hopper et al, 1979). Numerous in vivo stimulating agents have reputedly been used for the purpose of increasing the yield of primary macrophages for in vitro studies (reviewed by Harington et al, 1975), and it has been suggested that macrophage populations elicited by different biochemical agents may present a correspondingly different response following treatment with asbestos (Miller, 1978; Morgan and Allison, 1980). The alveolar macrophages would certainly be the most pertinent cell population for examination in this in vitro study, but the problem regarding the choice of the activational status

of the cell population still remains. It must be considered, therefore, that a permanent macrophage-like cell line may prove useful for routine use in this study.

A number of research groups have employed permanent cell lines for examination of fibre/cell interaction with regard to the involvement of fibre length; these have mainly comprised examination of the V79-4 (Brown et al, 1978), A549 (Brown et al, 1978), $P388D_1$ (Wade et al, 1980) and phagocytic ascitic cells (Beck and Tilkes, 1980). All of these systems have indicated that the longer fibres in a fibrous sample are more active in vitro than the shorter ones, and Brown et al (1978) have shown that those fibres longer than 6.5 μm possess the greatest activity. Brown et al (1978) have also suggested that results from their cell systems may be useful for the prediction of the in vivo carcinogenicity of a dust sample. The $P388D_1$ cells utilised by Wade et al (1976, 1980) have the advantage of being actively phagocytic and macrophage-like; this cell line has shown a similar susceptibility, as assessed by reduction in cell numbers, to asbestos treatment as macrophages (Wade et al, 1976), and a close association between cytotoxicity, fibres longer than 8 μm and fibre tumourigenicity has also been demonstrated (Wade et al, 1980; Lipkin, 1980). A number of other research groups have explored the usefulness of the $P388D_1$ cell line, Gormley et al (1979) have found the system adequate for in vitro examination of particulate dust material, Daniel and Le Bouffant (1980) also examined the $P388D_1$ cell response to treatment with particulate material, with the intention of comparing enzyme release from the permanent cell line and also from alveolar and peritoneal macrophages. They demonstrated that the results obtained for the two types of cell, ie permanent and primary cells, were similar and these workers recommended the use of the $P388D_1$ cell line for cytotoxicity assays because of its ready accessibility and reproducibility. Certainly the use of this cell line would appear to be advantageous, but before considering the replacement of the macrophage by the $P388D_1$ cells, some attention must be paid to the characteristics of this cell line which warrant the application of the term "macrophage-like".

4.1.3 "Macrophage-Like" Characteristics of the $P388D_1$ Cell Line.

The $P388D_1$ cell line was originally described by Dawe and Potter (1957), being derived from a lymphoid tumour (P388) originally induced in a DBA/2 mouse following treatment with methylcholanthrene. The cell line was repeatedly passaged through mice until it was observed, under tissue culture conditions, that the cell had acquired a number of macrophage-like properties. Koren et al (1975) described some of the more obvious macrophage-like characteristics of this cell line, these being the ability to phagocytose latex beads and adhere to glass, the presence of Fc and C3 receptors on the cell membrane, and the ability to exhibit a high effector activity in an antibody-dependent cell-mediated cytotoxicity system. The phagocytic capacity of the $P388D_1$ cell line was later confirmed by Goodell et al (1978) who demonstrated the requirement for adequate opsonisation and temperature conditions for efficient phagocytosis to occur. Lachman and Metzgar (1980) have also shown that $P388D_1$ cells and macrophages can release lymphocyte-activating factors (interleukin I) which are similar in nature. Thompson et al (1980) examined the activity of cyclic AMP phosphodiesterase, but reported that the degree of activity for $P388D_1$ cells and blood monocytes was different. It is known however, that cyclic nucleotide metabolism may alter during the process of activation (see Table 1.7), and it must therefore be considered that the cyclic AMP phosphodiesterase activity in the $P388D_1$ cells may reflect that of an activated macrophage rather than the less mature blood monocyte. It would appear therefore that the $P388D_1$ cell line has a number of properties which serve to recommend the application of the term "macrophage-like", and it must be considered that this cell type is highly qualified for the substitution of macrophages in a routine cytotoxicity assay.

4.1.4 Validation of the Choice of the $P388D_1$ Cell Line for Use in a Cytotoxicity Assay System.

Whilst the primary macrophage was the most relevant cell of choice for examination in this study, there were a number of disadvantages associated with its use:

i) The choice of activational status to be examined (Section 4.1.2).

ii) Should an appropriate activational status be chosen, it was not certain that macrophages could be routinely harvested without some variation in the activational status occurring from time to time due to the inadvertent exposure of the animals to localised pathogens or activating agents.

iii) An extensive loss of animal life would be associated with harvesting of adequate quantities of macrophages for the examination of a large number of dusts.

There were, however, a number of advantages associated with the use of a permanent cell line:

i) No loss of animal life.

ii) A guaranteed supply of unlimited quantities of cells.

iii) If the culture conditions were adequately maintained, there should be few problems regarding variation in the cell population compared to those seen with primary populations.

There were further advantages associated with the use of the $P388D_1$ cell line:

i) This cell is actively phagocytic and macrophage-like (Section 4.1.3). and therefore the obvious choice of cell to substitute in place of the macrophage.

ii) The cell has already proven useful in previous cytotoxicity studies (Gormley et al, 1979; Daniel and Le Bouffant, 1980).

iii) The cell line has already shown the same type of response as macrophages following treatment with asbestos (Wade et al, 1976).

iv) The cell line had already proven useful in a "fibre length" cytotoxicity study, and has been tentatively recommended for use as a toxicological predictor of in vivo tumourigenicity (Wade et al, 1980; Lipkin, 1980).

It was therefore concluded that the advantages associated with the use of a permanent cell line in a routine cytotoxicity assay were considerable, and would certainly solve a number of problems associated with the use of primary macrophages. The $P388D_1$ cell line was therefore the obvious cell of choice for use in this study. It must be borne in mind , however, that whilst this cell has a number of macrophage-like

characteristics, it is not a macrophage; thus whilst the results obtained from this study may serve to elucidate the mechanism of action of a fibrous dust sample with macrophages, they may not necessarily present the most precise picture.

4.1.5 Aims and Objectives.

The main aims of this study were:

i) To examine the cytotoxic effect of a variety of well-defined fibrous dust samples on $P388D_1$ cells, a cytotoxic effect being defined as an increase in membrane permeability to the vital dye trypan blue, an alteration in lactate production, an increase in the release of the cytoplasmic enzyme LDH and lysosmal enzyme glucosaminidase.

ii) To examine the relationship between the cytotoxic potential of a dust sample and the constituent fibre lengths.

iii) To assess the comparability of the $P388D_1$ cell system with other *in vitro* assay systems; in particular with reference to the possible toxicologically predictive nature of the assay system.

4.2 MATERIALS AND METHODS.

4.2.1 Fibre Samples Examined.

The fibrous samples examined in this study have already been described in Section 2.2.2 of this thesis. They were divided into two groups for study: Group 1 (Table 4.1) comprised all of the available fibrous samples with the exception of the WDC and milled chrysotile samples, although the original E WDC sample was included for comparison; Group 2 (Table 4.2) comprised all of the industrially prepared WDC samples, E milled chrysotile, and also UICC chrysotile for comparison. As one of the objectives of this study was to assess the role of fibre length in determining the cytotoxic potential of a dust, the fibre dimensions and fibre number content for each sample, where feasible, were established; the methods used to establish these as well as the results obtained have been described in Chapter 2 of this thesis.

4.2.2 The Routine Maintenance of $P388D_1$ Cells.

$P388D_1$ cells were routinely maintained and passaged in 75 cm^3 tissue culture flasks (Nunc). The volume of medium used in each flask was 30 mls and consisted of the Nutrient Mixture F10 (Ham, 1963) (Gibco-Biocult) supplemented with 10% heat-inactivated (56 °C for 30 mins) newborn calf serum (Gibco-Biocult). The antibacterial and antifungal agents used were Kanamycin Sulphate (100 µg/ml) (Winthrop Labs) and Fungizone (5 µg/ml) (ER Squibb and Sons Inc). The flasks were maintained at 37 °C in a katharometer-controlled CO_2 incubator (Gallenkamp Ltd); the cells were harvested from confluent flasks when they were required for use in cytotoxicity assays.

4.2.3 Cytotoxicity Assay Protocol.

The cytotoxicity assay utilised in this study was essentially similar to the one described by Gormley et al (1979), and a more detailed description of the various aspects of the system will be supplied in a later part of this section. In brief, the assay system comprised the use of $P388D_1$ cells which were seeded into culture plates

Table 4.1 List of Those Fibrous Samples Included in "Group 1" Series.

E ceramic fibre
UICC crocidolite
E UICC crocidolite
UICC amosite
E UICC amosite
E F amosite
SF amosite
E LF amosite
E tremolite
E UICC anthophyllite
E brucite
UICC chrysotile A
E UICC chrysotile A
SFA chrysotile
E F chrysotile
E PH chrysotile
E H chrysotile
E WDC

Table 4.2 List of Those Fibrous Samples Included in "Group 2" Series.

E milled chrysotile
E F WDC
E milled WDC
E heat-cleaned WDC
E unextracted WDC
E WDC
UICC chrysotile A

and allowed to adhere and spread. After 24 hrs the cells were examined, if they complied with various morphological and biochemical criteria they were treated with the dust samples. The assay described by Gormley et al (1979) was essentially designed for the study of particulate dust samples and they recommended the use of one concentration of dust (80 μg/ml), and the examination of any cytotoxic effects were carried out at one time point (48 hrs). This method was adapted for the purposes of the examination of fibrous samples (Wright et al, 1980; Gormley et al, 1980; Gormley et al, in press), so that two dust concentrations were examined (10 and 50 μg/ml) at two time points (24 and 48 hrs). It has been shown that a selective release of lysosomal enzymes can occur upon ingestion of a fibrous sample , and 24 hrs would appear to be an adequate time point for measurement of this phenomenon (Davies et al, 1974a); the dust concentrations 10 and 50 μg/ml were also employed by Davies et al (1974a). At the 24 and 48 hrs time points the degree of cytotoxicity was established by assessing: i) the cell membrane integrity by recording the ability of the cells to exclude the vital dye trypan blue (Gormley et al, 1979), ii) the cell membrane integrity by recording the loss of intracellular LDH (Gormley et al, 1979; Davies, 1980a, b), iii) the release of the lysosomal enzyme n-acetyl-β-D-glucosaminidase (Davies et al, 1974a; Gormley et al, 1979), iv) the metabolic activity of the cells by recording the lactate production (Beck et al, 1971).

4.2.3.1 Harvesting of $P388D_1$ Cells.

The $P388D_1$ cells were only harvested from those flasks that had achieved confluency. The culture medium was removed and replaced by 10 mls of phosphate buffered saline (PBS) (Gibco-Biocult). As $P388D_1$ cells cannot be removed from the surface of plastic by trypsinisation the flasks were agitated to encourage the detachment of the loosely attached cells. The harvested cells were poured into a sterile Universal container (Sterilin) and centrifuged at 200 g (MSE Super-Minor) for 10 mins. The cells were resuspended in PBS and the cell concentration assessed using an improved Neubauer haemocytometer. Cell viability was established using 0.5% trypan blue in saline (Gibco-Biocult); the cells were used for cytotoxicity assays if 95% or more of the cell population excluded trypan blue.

4.2.3.2 Plating Out $P388D_1$ Cells and Randomisation of Plates.

The harvested $P388D_1$ cells were resuspended to give a final concentration of 1 x 10^5 viable cells/ml of complete medium. The complete medium comprised F10 medium supplemented with 4% heat inactivated newborn calf serum and Kanamycin Sulphate (100 µg/ml) and Fungizone (5 µg/ml); this type of complete medium was used throughout the cytotoxicity assay. The suspended cells were placed in a 600 ml sterile glass bottle that had previously been siliconized with Repelcote (Hopkin and Williams). A 5 ml plastic pipette (Falcon) was used to dispense the cells, this pipette was filled with 5 mls of medium containing cells, and left for 10 mins. This procedure allowed the surface of the pipette to become coated with adherent cells, thereby reducing the possibility of cell loss due to further adherence of cells during dispensing.

50 mm plastic tissue culture dishes (Nunclon) were used for the assay, and they were numbered and stored on trays. 5 mls of suspended cells were added to each dish in numerical order, and each tray was carefully rocked from side to side to encourage the even dispersal of the cells across the plates. The plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 24 hrs, the plates were removed from the incubator and checked by light microscopy for evidence of bacterial or fungal contamination. To eliminate any effects due to the order in which the plates had been seeded, all of the plates were randomly renumbered and thereafter handled in their new numerical order.

4.2.3.3 Zero Time Viability and Biochemical Estimations.

In order to ascertain the suitability of the $P388D_1$ cells for further use in the assay, viability counts for 4 plates (Section 4.2.3.5a) and intracellular LDH and glucosaminidase levels for 4 plates (Sections 4.2.3.5c, d and e) were estimated. The remaining plates were used for the assay if they complied with the following criteria: i) if more than 95% of the cell population proved viable, according to their ability to exclude trypan blue, ii) if a mean count of 18 to 24 cells was observed per microscope field of view at a magnification of x 100 (this proved to be the optimum cell density for the suitable growth of cells

during the assay procedure (Gormley IP, personal communication)), iii) if the biochemical estimates showed intracellular levels of approximately 0.200 enzyme units of LDH and 0.0050 enzyme units of glucosaminidase per plate.

4.2.3.4 The Addition of Dust Samples to the Culture Dishes.

10.0 mg of each dust sample were suspended in 10 mls of PBS and placed in the ultrasonic bath for 2 mins to ensure the adequate dispersal of the sample (previously described in Section 2.2.4.2). The concentration of each sample was adjusted to give a final concentration of either 10 or 50 μg/ml of complete medium. The particulate controls TiO_2 and DQ_{12} were prepared in a similar manner, but adjusted to give a final concentration of 80 μg/ml for TiO_2 and 20 μg/ml for DQ_{12} in complete medium (Gormley et al, 1979; Robock, 1973). Care was taken when preparing the WDC samples to ensure that a maximum dispersal of fibres was obtained for each sample.

Prior to the addition of dust samples, the medium was removed from the plates and each plate was washed with 5 mls of PBS to remove any cell debris. The plates were treated with 5 mls of one of the following medium:

i) dust-free complete medium ie untreated control.
ii) medium containing 80 μg/ml of TiO_2 - "negative" control.
iii) medium containing 20 μg/ml of DQ_{12} - "positive" control.
iv) medium containing 10 μg/ml of test dust.
v) medium containing 50 μg/ml of test dust.

14 plates were established for each of conditions i) to v); 7 plates were examined at the 24 hrs time point and 7 plates at 48 hrs following incubation at 37 °C in a humidified atmosphere of 5% CO_2 in air. In addition 20 mls of complete medium were incubated in similar conditions in a Universal container; after 24 and 48 hrs this medium was used as a "background" control in the biochemical assays.

4.2.3.5 Cytotoxicity Assays.

After 24 and 48 hrs incubation the following assays were performed for each of the treatments i) to v): i) cell viability estimates (3 plates), ii) biochemical measurements comprising an assessment of LDH and glucosaminidase levels in cells and medium, and an assessment of lactate levels in the medium (4 plates).

a) Viability Count.

Before assessing cell viability, all plates were coded to ensure that no bias occurred during the cell counting procedure. The medium was removed and each plate washed gently with PBS to remove cell debris, non-adherent cells and excess dust. The $P388D_1$ cells were treated with 1 ml of 0.5% trypan blue in saline (Gibco-Biocult) for 1 min and the excess removed with a Pasteur pipette. A 24 x 32 mm coverslip (Chance Propper Ltd) was carefully placed over the cells and the plate examined at a magnification of 100 x under a Nikon light microscope. 10 randomly chosen microscope fields were examined across each plate and the number of both live and dead cells noted for each field. A cell was termed dead if it was unable to exclude trypan blue (Paul, 1975). The number of live cells per plate was expressed in terms of the mean count per plate. The mean count for each of the 3 plates was averaged to produce the final number of live cells per treatment; the SD for this figure rarely exceeded 15%.

b) Phagocytic Capacity of $P388D_1$ Cells.

In order to establish that the $P388D_1$ cells had undergone phagocytosis during each experiment, the number of cells that had phagocytosed TiO_2 particles were recorded. The result was never less than 95%.

c) Harvesting of Cells for Biochemical Assays.

To carry out the biochemical assays it was necessary that the culture medium and also the $P388D_1$ cells should be harvested from each experimental culture plate. The medium from each plate was poured

into a plastic Universal tube and centrifuged at 600 g for 10 mins to encourage the sedimentation of the majority of the fibres and cell debris. 2 mls of 0.1% Triton-X detergent (Koch-Light) in distilled water were added to each plate to cause cell lysis. After 5 mins the cells were scraped from the surface of the dish with a rubber policeman; the resulting suspension was placed in a 10 ml plastic test tube (Sterilin) and centrifuged at 500 g for 10 mins to encourage the sedimentation of any cell fragments and dust particles. The following biochemical assays were carried out on the cell and medium supernatants. All chemical reagents, unless otherwise stated, were of Analar grade and were obtained from Sigma, BDH or Fisons Chemicals; all molarities and pH values were established at 22 °C.

d) Lactate Dehydrogenase (LDH) Assay.

LDH activity was measured in cells and medium using the method of Wróblewski and LaDue (1955). In summary, the method for estimation of LDH utilised the conversion of pyruvate to lactate by LDH, the reaction is accompanied by the oxidation of NADH to NAD^+ and thus the decrease in NADH levels can be used to estimate the activity of LDH.

The reaction mixture comprised the following reagents, which were gently inverted in an RT30 tube (Sterilin):
0.4 mls test sample (cell lysate, medium supernatant or medium control)
2.7 mls of sodium pyruvate (Sigma) at a concentration of 0.072 mg/ml of 0.05 M sodium phosphate buffer pH 7.5.
50 µl of NADH (8 mg/ml of distilled water) (Sigma).

The rate of oxidation of NADH at 37 °C in 30 sec was established using an SP30 spectrophotometer (Pye Unicam) set to read absorbance at a wavelength (λ) of 340 nm for NADH. In order to ensure that all reagents were functioning correctly, a Boehringer Mannheim standard serum control (Precinorm E) was used according to the recommendations supplied with the test kit.

The absorbance values for both cell lysate and medium may be converted to enzyme units using the Beer-Lambert Law (Bergmeyer and Gawehn, 1978):

$$C = \frac{A}{\varepsilon \times P}$$

where: C = concentration in moles/litre
ε = extinction coefficient = 6220
P = path length = 1 cm
A = absorbance

thus for NADH:

$$C = \frac{1}{6.22} \text{ μmoles/ml}$$

In order to account for the dilutions involved in establishing the reaction mixture:

$$C = \frac{VI \times VC}{\varepsilon \times SV}$$

where: C = concentration in μmoles/plate
VI = volume of incubation mixture ie 3.15 mls
VC = total volume available in culture plate ie 5 mls of medium or 2 mls of cell lysate
ε = extinction coefficient
SV = volume of test sample ie 0.4 mls

The absorbance value may be converted to enzyme units by multiplying by 2.532 for the cell lysate and 6.330 for the medium. One enzyme unit of LDH was therefore defined as the quantity of enzyme causing the conversion of 1 μmole of NADH to NAD^+ at 37 °C at pH 7.5 in 30 sec. The final value for the medium supernatant was obtained by subtracting the value obtained for the medium control, this effectively removed any extraneous levels of LDH due to the presence of newborn calf serum.

e) N-acetyl-β-D-glucosaminidase Assay.

The levels of the enzyme glucosaminidase were estimated in both medium and cell lysate according to the method of Woolen et al (1961); this method has been adapted at the IOM by substituting the borate buffer with 0.2 M glycine buffer. The basis of the assay system involved an estimation of the cleavage of p-nitrophenol-n-β-D-glucosaminide by the enzyme glucosaminidase. The procedure comprised the incubation of 0.2 mls of test sample (either medium supernatant, medium control or cell lysate) with 1.2 mls of buffered glucosaminide (0.765 mg of glucosaminide (Sigma) per ml of 0.1 M citric acid buffer pH 4.5) for 1 hr at 37 °C in an RT 30 tube. The reaction was terminated by the

addition of 1.2 mls of 0.2 M glycine buffer pH 10.4. A cell control tube was also established by mixing 0.2 mls of cell lysate with 1.2 mls buffered glucosaminide and 1.2 mls glycine buffer; the mixture was incubated at 37 °C for 1 hr. The absorbance values for free para-nitrophenol were established for each tube using an SP30 spectrophotometer set to read absorbance at a λ of 400 nm. Before reading the cell lysate or medium supernatant tubes the spectrophotometer was set to zero using the appropriate cell or medium control, this procedure effectively removed any absorbance due to the buffers or serum.

The absorbance values for both cell lysate and medium supernatant were converted to enzyme units using the Beer-Lambert Law as described in the previous section, where:

$$C = \frac{VI \times VC}{\varepsilon \times SV \times T}$$

where: C = concentration in μmoles/plate
VI = total volume of incubation mixture ie 2.6 mls.
VC = total volume of solution on culture plate ie 5 mls for medium and 2 mls for cell lysate
ε = extinction coefficient for p-nitrophenol ie 18.8.
SV = sample volume ie 0.2 mls.
T = incubation time ie 60 mins.

The absorbance values may therefore be converted to enzyme units by multiplying by 0.0231 for the cell lysate and 0.0576 for the medium supernatant. One enzyme unit was defined as the amount of enzyme glucosaminidase capable of releasing one μmole of p-nitrophenol from the substrate per minute per plate at 37 °C and pH 4.5.

f) Lactate Assay.

L-(+)-lactate levels in the medium only were measured using the method of Gutmann and Wahlefeld (1974). The assay system examined the conversion of the lactate to pyruvate by LDH, and involved a reduction of NAD^+ to NADH, therefore an increase in NADH could also be

used as a measure of the conversion of the lactate present in the medium. All of the medium samples were deproteinised by treating 1.8 mls of sample with 0.2 mls of 30% (v/v) perchloric acid followed by 0.2 mls of 3 M potassium carbonate. The resulting precipitate was removed by centrifugation at 500 g for 15 mins. The deproteinised medium samples were examined for the presence of lactate by incubating:

0.1 mls of sample (test medium or medium control)
2.3 mls of semicarbazide/glycine buffer (0.5 M semicarbazide hydrochloride in 0.5 M glycine buffer pH 10.4)
0.2 mls of NAD (Sigma) (17.9 mg NAD/ml of distilled water).
20 µl LDH (10 mg/ml) (Sigma).

The reaction mixture was incubated in an RT30 tube at 37 °C for 1 hr. A Boehringer-Mannheim Precipath S control was incorporated into the assay system. The absorbance values for NADH were read spectrophotometrically at a λ of 340 nm; the spectrophotometer was set to zero using the medium control tube.

The absorbance values for medium supernatant were converted to µg of lactate per plate using the Beer-Lambert Law as described previously where:

$$C = \frac{VI \times VC \times DS \times MW}{\epsilon \times SV \times DR}$$

where:
C = concentration ie µg/plate
VI = volume of incubation mixture ie 2.62 mls.
VC = volume on plate ie 5 mls.
DS = deproteinisation sample volume ie 1.8 mls.
ϵ = extinction coefficient ie 6.220
SV = sample volume ie 0.1 mls.
DR = deproteinisation reaction volume ie 2.2 mls.
MW = molecular weight of lactate ie 89.

The level of the lactate per plate was therefore estimated by multiplying the absorbance value by 1534.

4.2.3.6 The Treatment and Presentation of the Data from the Cytotoxicity Assays.

The following results were calculated for each dust treatment at each concentration and time point, and were represented as a mean of the appropriate number of replicates.

cell viability — (mean of 3 replicates ± SD)

LDH in cells
LDH in medium
LDH - total per plate
% LDH released per plate

glucosaminidase in cells
glucosaminidase in medium
glucosaminidase in total per plate
% glucosaminidase released per plate

lactate in medium

— (mean of 4 replicates ± SD)

In order to remove any effects due to the presence of a "non-toxic" dust, both the viability and the biochemical data for each test dust were expressed as a percentage of the result obtained for the TiO_2 "non-cytotoxic" control dust ie:

$$\% \text{ viablity} = \frac{\text{result obtained for test dust}}{\text{result obtained for } TiO_2 \text{ control}} \times 100$$

Each experiment was repeated on at least 3 separate occasions. The data were finally expressed as a mean value for the 3 experiments ± SD.

4.2.4 Statistical Examination of Cytotoxicity Data and Examination of the Relationship Between Viability, Enzyme Release and Fibre Length.

The Student T test (Bailey, 1974) was used to demonstrate the statistical significance of the differences obtained between the cytotoxicity results for the different dust treatments.

In order to establish the relationship between cellular viability and the enzyme levels, all of the values for each measured parameter (combining the data from Group 1 and Group 2 dusts) were plotted and compared, 2 parameters being compared at one time point, for example viability versus intracellular LDH at 10 μg/ml for 24 hrs. From preliminary plots of selected assays a linear relationship appeared to be the most reasonable, and correlation coefficients were calculated to show the level of significance for each association (Bailey, 1974). All associations were calculated using the computer-based Genstat Statistical Package (compiled by Lawes Agricultural Trust, Rothamstead Experimental Station). For those occasions when the associations proved non-linear, the relationship was examined in greater depth by converting the individual values for each parameter to the $\log_e$ equivalent, and correlation coefficients for the associations were calculated as described previously.

A total of 13 fibrous dusts were selected for the comparison of fibre length and cytotoxicity data; these dusts were selected because of their purity and lack of contamination with unidentified non-fibrous material, and these are listed in Table 4.3. The viability and enzyme data for these dust samples were compared, as described previously, to establish the relationship between cell viability and enzyme levels. To examine the relationship between fibre length and cytotoxicity, the viability and enzyme data were compared with the number of fibres greater than each stated length (from 0 to 20 μm in 1 μm steps) for each given dust population, for example : % viability versus number of fibres greater than 1 μm in length at 10 μg/ml for 24 hrs. From preliminary plots of selected assays a linear relationship generally appeared to be the most reasonable, and correlation coefficients for each association were calculated using the Genstat Statistical Package. In the case of those relationships that proved non-linear, the individual values for each parameter were converted to the $\log_e$ equivalent and compared as described previously.

4.2.5 Fixation and Staining of Cells for Light Microscopy.

To examine the morphology of the $P388D_1$ cells, the following procedure for fixation and staining was carried out:

Table 4.3 List of Fibrous Samples Selected for Comparisons Between Fibre Length and Cytotoxicity Data.

E ceramic fibre
UICC crocidolite
E UICC crocidolite
UICC amosite
E UICC amosite
SF amosite
E LF amosite
E tremolite
E UICC anthophyllite
E brucite
UICC chrysotile A
E UICC chrysotile A
SFA chrysotile

i) Plates were washed in PBS.
ii) Cells were fixed for 2 mins with 10% methanol.
iii) Cells were stained with May-Grunwald stain for 2 mins.
iv) Cells were stained with 10% Giemsa for 10 mins.
v) Plates were washed with distilled water to remove excess stain.
vi) Plates were air-dried.
vii) The cells were mounted in glycerol 5 mins prior to optical observation and photography using a Nikon light microscope.

Photographs of unstained and unfixed $P388D_1$ cells were taken *in situ* using a Nikon inverted light microscope.

4.2.6 Experiments to Assess the Occurrence of $P388D_1$ Cells With More Than One Nucleus.

A morphological examination of $P388D_1$ cells following treatment with asbestos fibres, showed the occurrence of a number of cells with more than one nucleus (see results Section 4.3.1). The following experiments were therefore carried out in an attempt to try and quantify this phenomenon.

4.2.6.1 Cell Culture.

The $P388D_1$ cells were harvested as described previously (Section 4.2.3.1), and the cell concentration adjusted to 0.33×10^5 cells/ml of F10 containing 4% heat inactivated newborn calf serum (complete medium). 3 mls of cell suspension were seeded in each 35 mm tissue culture dish (Nunc) and incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 24 hrs the cultures were washed with PBS and the complete medium replenished. 3 plates were fixed and stained as described in Section 4.2.5.

4.2.6.2 Treatment of Plates With Dust.

The following dust samples were examined:
TiO_2 - "non-cytotoxic" particulate control
DQ_{12} - "cytotoxic" particulate control

Hetton coal dust - coal sample of low toxicity (IP Gormley, personal communication).

Samples of UICC crocidolite, amosite and chrysotile A.

10 mgs of each sample were ultrasonicated in 10 mls of medium for 2 mins as described previously (Section 2.2.4.2). Aliquots of either 20 µl or 100 µl of the dust suspension were added to each plate to give a final concentration of 20 µg or 100 µg of dust per plate. Triplicate plates were established for each condition, as well as triplicate untreated control plates. The dishes were agitated to encourage the even dispersal of the dust. After 24 or 48 hrs of incubation at 37 °C in a humidified atmosphere of 5% CO_2 in air, the cells were fixed and stained as described previously (Section 4.2.5).

4.2.6.3 Optical Estimation of Cells With More Than One Nucleus.

Each plate was mounted in glycerol 5 mins prior to examination. At a magnification of 100 x, 5 randomly chosen fields of view were scored for each plate, and the number of cells containing i) one nucleus or ii) more than one nucleus were noted. The 5 values of i) and ii) from each plate were averaged to give a mean value for each cell type.

4.2.6 Presentation of Data and Statistical Analysis.

The results were expressed as the percentage of cells containing more than one nucleus, and the results for each condition were presented as a mean of the values from the triplicate plates. Each experiment was repeated on three separate occasions. The Student T test (Bailey, 1974) was used to estimate the statistical significance of the differences obtained between the different dust treatments.

4.3 RESULTS.

4.3.1 The Morphological Appearance of P388D$_1$ Cells: Control Cells and Those Treated With Dust Samples.

The characteristic appearance of control P388D$_1$ cells following 48 hrs in culture with complete medium alone is shown in Figures 4.1 and 4.5. The cells had adhered to and completely spread onto the plastic surface of the Petri dish (Figure 4.1), and a number of cells were observed that were vacuolated and "foamy" in appearance (Figure 4.5). The treatment of P388D$_1$ cells with the non-cytotoxic particulate control TiO_2 (80 μg/ml) resulted in an extensive phagocytosis of the dust particles at the 48 hrs time point (Figure 4.6); the occurrence of phagocytosis was manifest by the clearance of dust particles to form a dust-free zone around each cell. In contrast, treatment of the cells with the cytotoxic positive control DQ_{12} (20 μg/ml) resulted in the occurrence of a number of cells that had become shrunken and pyknotic in appearance (Figure 4.7). The exposure of P388D$_1$ cells to fibrous samples of either UICC crocidolite, amosite or chrysotile (50 μg/ml) resulted in the cells attempting to ingest fibres that varied considerably in length, some of the fibres being longer than the cells themselves (Figures 4.2 to 4.4). Ingestion of the amphibole samples crocidolite or amosite, did not result in any apparent deterioration of the intracellular components (Figure 4.2, 4.3 and 4.8), although an increase in the number of cells containing more than one nucleus following 48 hrs of treatment with crocidolite (Figure 4.8) and amosite (data not shown) was observed. A different response following treatment with chrysotile was noted, after 48 hrs a marked reduction in cell number per field of view (Figure 4.4), compared to the untreated control (Figure 4.1), was observed, and the majority of the cells had become either swollen or pyknotic in appearance (Figure 4.4 and 4.9).

4.3.2 The Long Term Reproducibility of the P388D$_1$ Cell Assay System: A Comparison of the Results for Untreated and DQ_{12} Treated Controls from Experiments Examining Group 1 and Group 2 Dust Samples.

In order to assess the long term reproducibility of the P388D$_1$ cell assay system over a period of 2 years, the viability and enzyme release

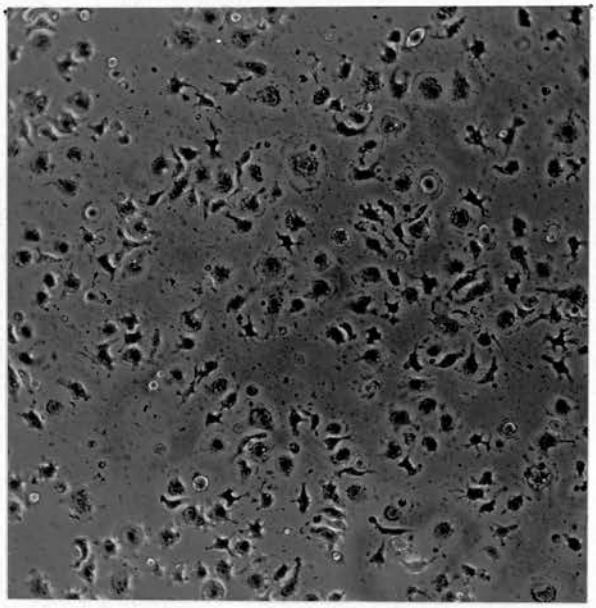

Figure 4.1
Control P388D$_1$ Cells 48 Hrs After Culture Without Dust.
(Phase Contrast Microscopy x 150).

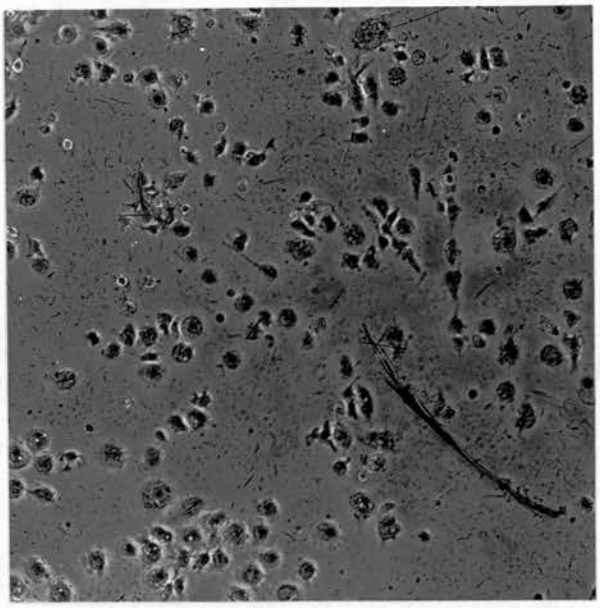

Figure 4.2
P388D$_1$ Cells 48 Hrs After Treatment With UICC Crocidolite (50 μg/ml).
(Phase Contrast Microscopy x 150).

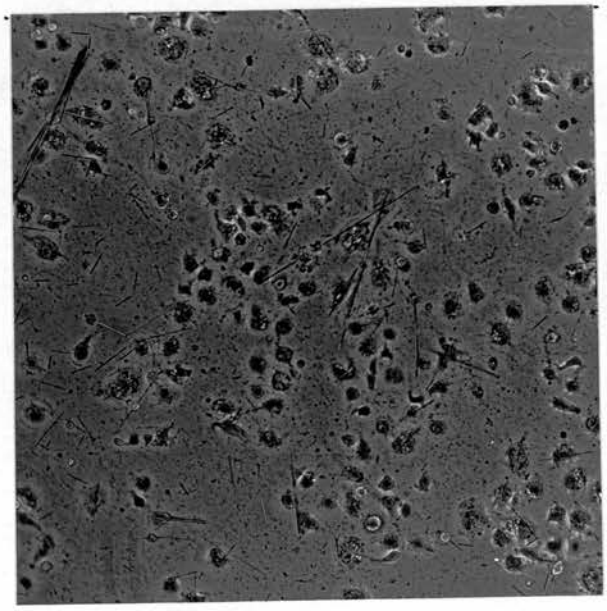

Figure 4.3
P388D$_1$ Cells 48 Hrs After Treatment With UICC Amosite (50 μg/ml).
(Phase Contrast Microscopy x 150).

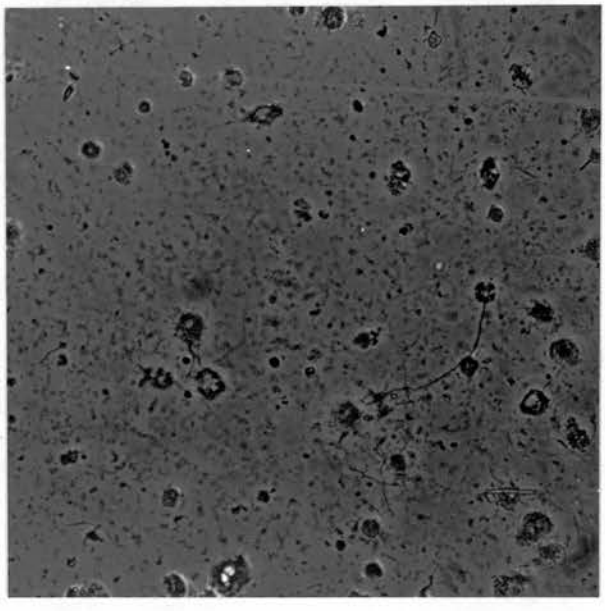

Figure 4.4
P388D$_1$ Cells 48 Hrs After Treatment With UICC Chrysotile (50 μg/ml).
(Phase Contrast Microscopy x 150).

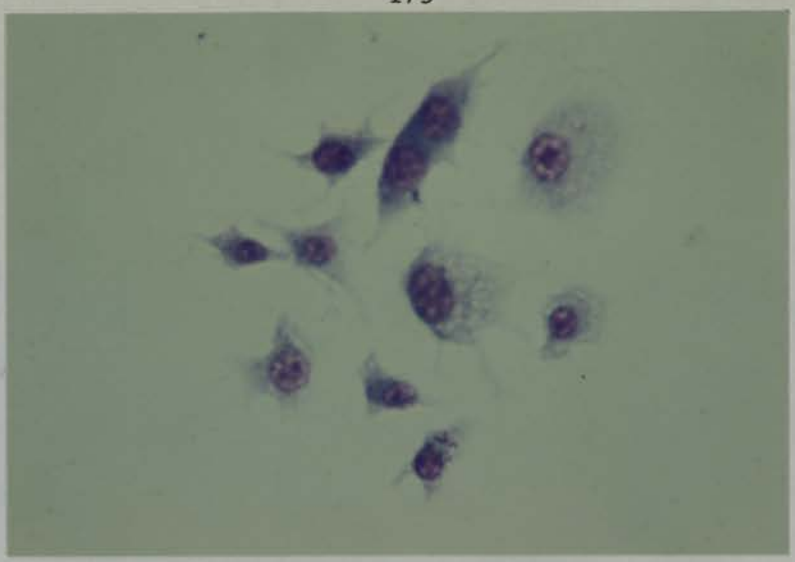

Figure 4.5 $P388D_1$ Cells 48 Hrs After Culture Without Dust. (x 350).

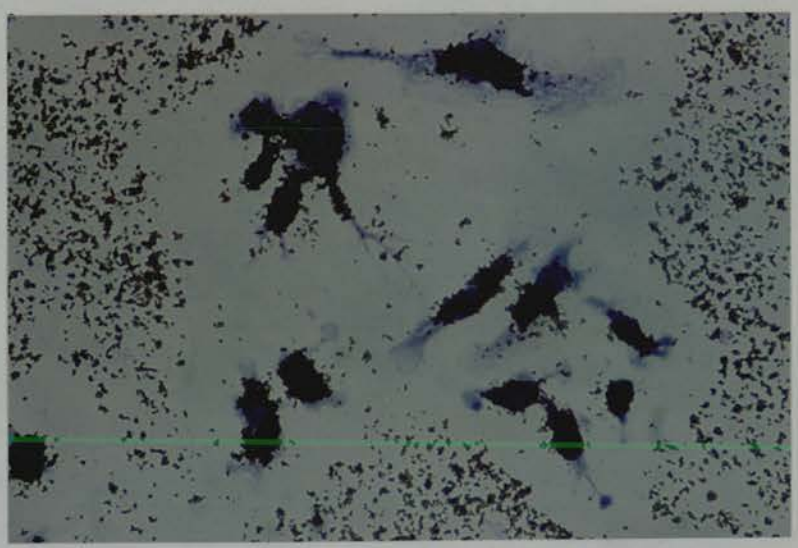

Figure 4.6 $P388D_1$ Cells 48 Hrs After Treatment With TiO_2 (80 µg/ml). (x 350).

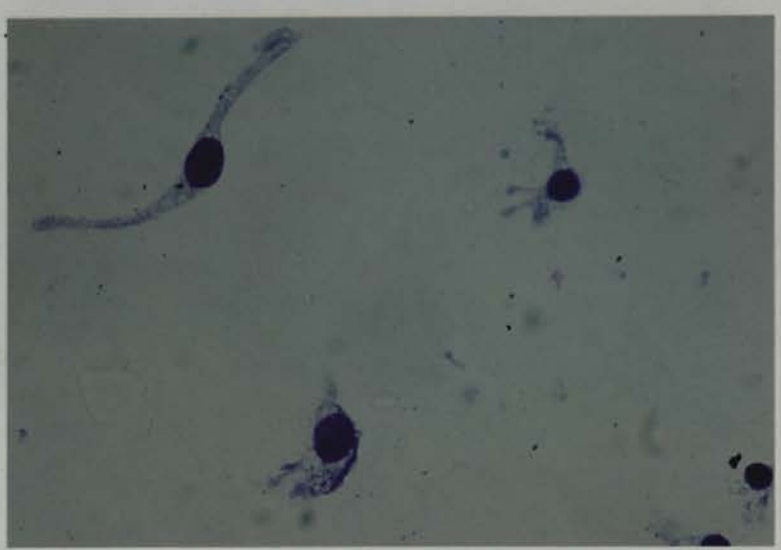

Figure 4.7 $P388D_1$ Cells 48 Hrs After Treatment With DQ_{12} (20 µg/ml). (x 350).

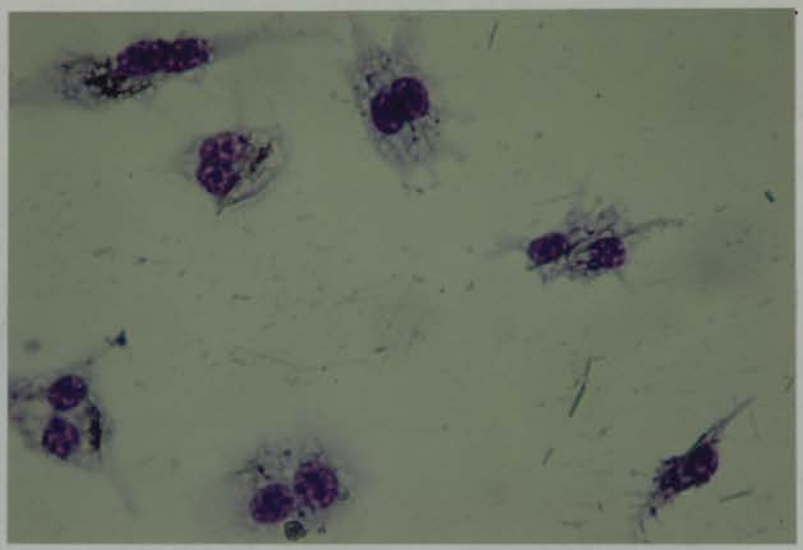

Figure 4.8 $P388D_1$ Cells 48 Hrs After Treatment With UICC Crocidolite (50 µg/ml). (x 350).

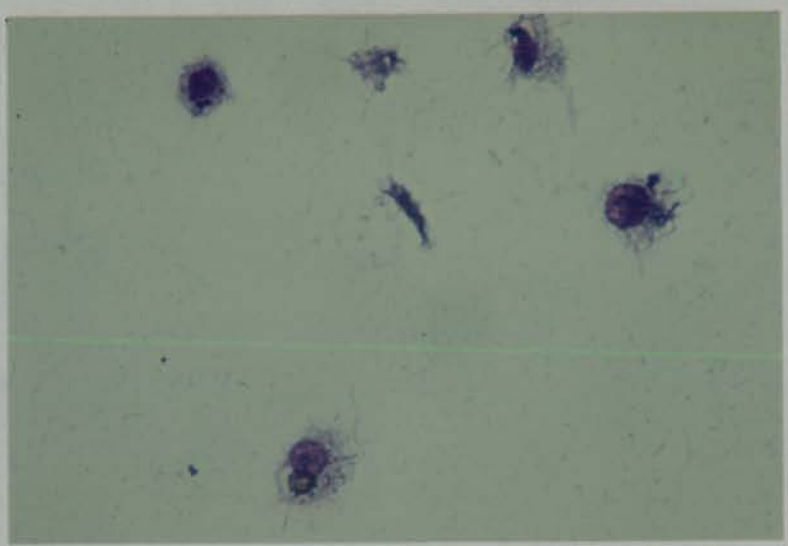

Figure 4.9 $P388D_1$ Cells 48 Hrs After Treatment With UICC Chrysotile (50 µg/ml). (x 350).

data from the untreated and DQ_{12} controls for each of Group 1 and Group 2 dust samples were compared (Table 4.4). 2 sets of data can be seen in each box for each assay condition, the top line of each box refers to the controls for the Group 2 WDC samples. The figures have been produced by percentaging each experimental value against the value for the TiO_2 control, and each figure is a mean of all of the control values obtained for Group 1 dusts (32) and Group 2 dusts (9). The pattern of enzyme release from the controls will also be examined prior to the study of the effects of mineral fibres.

4.3.2.1 The Reproducibility of the Control Values.

In general, a close correlation was observed between the 2 sets of data for the untreated controls (Table 4.4); those sets of data which showed a statistically significant difference have been marked with an asterisk, and significant differences ($p<0.01$) were observed between the values for the intracellular glucosaminidase levels, the total level of glucosaminidase on the plates, and also the percentage of glucosaminidase released into the medium at both the 24 and 48 hrs time points. In the case of DQ_{12}, the pairs of results for each parameter were consistently similar, and no significant differences were observed between the Group 1 and Group 2 results. This finding would suggest that a satisfactory level of reproducibility for the effect of dust samples on $P388D_1$ cells may be attained, thereby allowing a direct comparison of data for different dust samples from separate experiments.

4.3.2.2 Percentage Viability.

The untreated control samples consistently showed a higher viability than the TiO_2 control at 24 and 48 hrs (Table 4.4), whereas those cells treated with DQ_{12} showed a lower viability at 24 and 48 hrs than those exposed to TiO_2 ($p<0.001$).

4.3.2.3 Lactate Production.

The production of lactate by the untreated controls was similar to that observed for the TiO_2 treated cells; the DQ_{12} treatment,

Table 4.4 A Comparison of the Values Obtained for the Untreated and DQ_{12} Treated Controls During Examination of Group 1 and Group 2 Series of Dusts.

		24 Hr Time Point		48 Hr Time Point	
		Untreated Cells	DQ_{12} Treated Cells	Untreated Cells	DQ_{12} Treated Cells
	% Viability	106.4 ± 9.9 105.2 ± 10.6	54.4 ± 16.9 45.2 ± 9.9	115.9 ± 16.5 113.3 ± 14.0	37.5 ± 13.8 28.7 ± 6.9
	% Lactate Production	113.9 ± 47.8 100.8 ± 18.5	96.8 ± 24.8 110.9 ± 23.7	106.2 ± 30.8 100.7 ± 23.7	75.4 ± 17.5 84.8 ± 23.7
Lactate Dehydrogenase	In Cells	105.0 ± 9.4 111.4 ± 20.5	65.7 ± 17.3 57.9 ± 9.8	112.4 ± 16.0 112.1 ± 7.3	51.7 ± 12.9 41.7 ± 10.4
	Total Per Plate	108.3 ± 13.1 115.9 ± 28.4	100.8 ± 11.6 105.2 ± 7.3	110.2 ± 17.0 104.1 ± 15.9	101.1 ± 10.8 90.9 ± 25.5
	In Medium	284.6 ± 690.3 58.5 ± 40.2	1526.0 ± 2797.9 1589.6 ± 206.5	133.1 ± 158.1 100.2 ± 49.9	521.0 ± 508.5 575.3 ± 659.1
	Overall % Released Into Medium	146.9 ± 178.7 64.4 ± 69.4	407.6 ± 410.5 603.5 ± 581.8	114.9 ± 122.7 85.7 ± 57.4	475.4 ± 405.8 685.6 ± 86.8
Glucosaminidase	In Cells	*141.8 ± 40.2 207.1 ± 32.1	92.6 ± 32.8 77.6 ± 18.1	*172.2 ± 65.3 291.9 ± 110.8	72.5 ± 27.8 54.8 ± 23.8
	Total Per Plate	*129.3 ± 24.1 170.8 ± 25.2	96.8 ± 18.9 98.8 ± 10.7	*128.9 ± 27.2 162.5 ± 23.0	97.1 ± 17.8 100.9 ± 35.8
	In Medium	118.3 ± 29.9 128.0 ± 31.9	122.2 ± 29.4 112.1 ± 22.0	108.7 ± 22.9 112.8 ± 26.4	79.2 ± 17.9 83.1 ± 12.9
	Overall % Released Into Medium	*90.8 ± 18.2 76.7 ± 10.0	135.7 ± 31.2 113.6 ± 36.9	*83.9 9.4 66.6 5.5	104.1 ± 14.9 111.5 ± 12.8

Results are expressed as a percentage of the value obtained for the TiO_2 control and are mean values ± SD.

The figure at the top of each box refers to the control value for Group 1 dusts, the lower figure refers to Group 2.

* Denotes that the 2 figures in the marked box have a difference that is statistically significant ($p<0.01$).

however, had caused a significant decrease in lactate production by 48 hrs ($p<0.001$).

4.3.2.4 LDH Levels.

The untreated control cells showed a slightly greater intracellular level of the enzyme LDH than the TiO_2 control, although treatment with DQ_{12} caused a significant decrease of intracellular LDH at both 24 and 48 hrs time points ($p<0.001$). The total levels of LDH (incorporating both the intracellular and extracellular levels) per plate for both the untreated and DQ_{12} treated controls did not differ significantly from 100% at either the 24 or 48 hrs time points. Unfortunately, the values for the levels of LDH in the medium and also the overall percentage of LDH released into the medium were not readily interpretable because of the very large standard deviations around the mean values for these variables. It was observed that TiO_2 induced the release of only very small quantities of LDH into the medium, thus the quantity of LDH released from the cells by virtue of their exposure to a cytotoxic dust, when presented as a percentage of the quantity released from the TiO_2 treated cells, resulted in the occurrence of large inter-experimental differences.

4.3.2.5 Glucosaminidase Levels.

With respect to the levels of glucosaminidase measured for the experimental controls (Table 4.4), the untreated control cells were shown to consistently possess higher quantities of intracellular glucosaminidase than the TiO_2 treated controls, and DQ_{12} caused a decrease of intracellular glucosaminidase that was significantly lower than the level observed for TiO_2 at 48 hrs ($p<0.001$). The loss of intracellular glucosaminidase did not always correspond to the levels of the enzyme that were shown to be present in the culture medium of the appropriate plates. The quantity of glucosaminidase present in the medium of the untreated control plates never dropped below the value observed for the TiO_2 plates; treatment with DQ_{12} resulted in a lower quantity of glucosaminidase in the culture medium than was observed for either the untreated control or TiO_2 treated plates at the 48 hrs time point ($p<0.001$). With regard to the total amount of

glucosaminidase present in the culture plates, the untreated control plates apparently possessed a greater total level of the enzyme than those plates treated with TiO_2 at the 24 hrs and 48 hrs time points, although treatment with DQ_{12} resulted in an enzyme level that was similar to that observed following the TiO_2 treatment. The overall percentage of glucosaminidase per plate released into the medium by the untreated control cells was generally lower than the percentage released following TiO_2 treatment at 24 and 48 hrs, there was, however, no significant difference observed between the percentage of the enzyme released following either TiO_2 or DQ_{12} treatment.

4.3.3 General Trends in Altered Viability and Enzyme Release Patterns of $P388D_1$ Cells Following Treatment with Group 1 Fibrous Dusts.

The effect of treatment of $P388D_1$ cells with 18 different fibrous samples (Group 1 dusts are described in Table 4.1) for 24 and 48 hrs has been assessed by measuring cell viability, the lactate production, LDH and glucosaminidase release. Each result has been expressed as a percentage of the value obtained for the experimental TiO_2 control dust; the results are presented in Tables 4.5 to 4.14, each value is a mean of at least three separate experiments ± SD. In order to aid the interpretation of the data, the results have also been displayed in the form of histograms; this method of presentation also allows an easy visualisation of the overall trends in either altered viability or enzyme levels caused by the various dust samples. The sequential order in which the dusts have been placed in the histograms is directly related to their ability to cause a decrease in cell viability (assessed by the trypan blue exclusion test) after exposure to 50 μg/ml of dust for 48 hrs. The SDs have been omitted from the histograms, but these are given in Tables 4.5 to 4.14 if required.

4.3.3.1 Percentage Viability.

The percentage viability of the $P388D_1$ cells following treatment with 10 or 50 μg/ml of dust is shown in Figures 4.10 and 4.11. It can be seen that a dust concentration of 50 μg/ml caused a greater loss of viability than the 10 μg/ml concentration at both time points, and a greater degree of cytotoxicity, as assessed by viability, was

Table 4.5 Percentage Viability of $P388D_1$ Cells Following Treatment With Group 1 Dusts.

Treatment	24 Hrs		48 Hrs	
	10 μg/ml	50 μg/ml	10 μg/ml	50 μg/ml
E Ceramic	114.3 ± 8.2	107.9 ± 9.0	108.0 ± 9.5	105.4 ± 1.3
UICC Crocidolite	102.9 ± 9.0	85.9 ± 8.2	98.7 ± 9.9	62.6 ± 10.2
E UICC Crocidolite	104.1 ± 7.8	92.7 ± 6.6	117.1 ± 13.4	75.3 ± 10.7
UICC Amosite	101.2 ± 5.5	90.1 ± 5.8	99.2 ± 13.9	66.7 ± 18.9
E UICC Amosite	94.1 ± 14.8	81.5 ± 10.0	87.3 ± 7.4	74.4 ± 5.6
E F Amosite	88.7 ± 8.3	82.9 ± 15.0	88.5 ± 7.6	66.0 ± 7.7
SF Amosite	101.7 ± 5.3	95.7 ± 7.6	98.1 ± 8.7	103.0 ± 4.3
E LF Amosite	92.3 ± 3.4	86.4 ± 6.3	87.4 ± 1.9	65.7 ± 5.7
E Tremolite	101.4 ± 2.8	75.1 ± 5.2	94.2 ± 3.1	44.5 ± 9.6
E UICC Anthophyllite	104.0 ± 12.9	75.3 ± 15.0	96.9 ± 6.7	46.4 ± 27.8
E Brucite	77.2 ± 10.5	40.3 ± 13.4	73.6 ± 12.7	24.8 ± 4.6
UICC Chrysotile	71.0 ± 17.9	35.1 ± 21.5	29.8 ± 15.5	9.0 ± 8.5
E UICC Chrysotile	96.4 ± 6.0	59.9 ± 18.9	67.9 ± 9.7	30.4 ± 8.4
SFA Chrysotile	82.8 ± 16.2	53.9 ± 15.0	44.5 ± 20.4	15.6 ± 10.9
E F Chrysotile	79.2 ± 14.6	49.7 ± 12.0	72.0 ± 8.0	20.2 ± 7.0
E PH Chrysotile	95.6 ± 3.8	70.1 ± 12.7	82.0 ± 10.1	25.8 ± 13.0
E H Chrysotile	100.9 ± 14.8	84.2 ± 10.4	93.0 ± 14.0	61.1 ± 5.0
E WDC	43.5 ± 12.2	31.8 ± 14.8	13.1 ± 4.9	8.5 ± 3.2
Untreated Control	106.4 ± 9.9		115.9 ± 16.5	
DQ_{12}	54.4 ± 16.9		37.5 ± 13.8	

Results are expressed as a percentage of the TiO_2 control value; each value is a mean of at least 3 experiments ± SD.

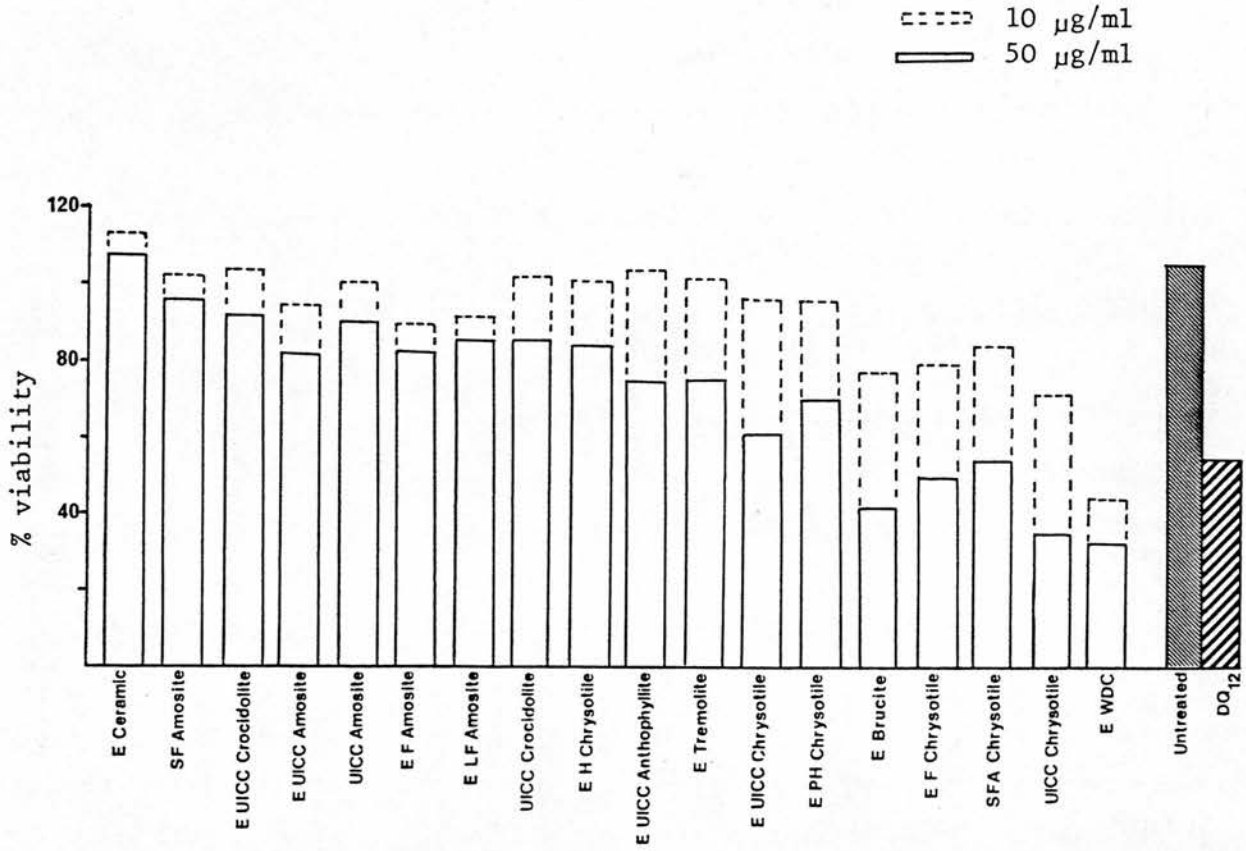

Figure 4.10 Percentage Viability 24 Hrs Following Treatment of $P388D_1$ Cells With Group 1 Dusts.

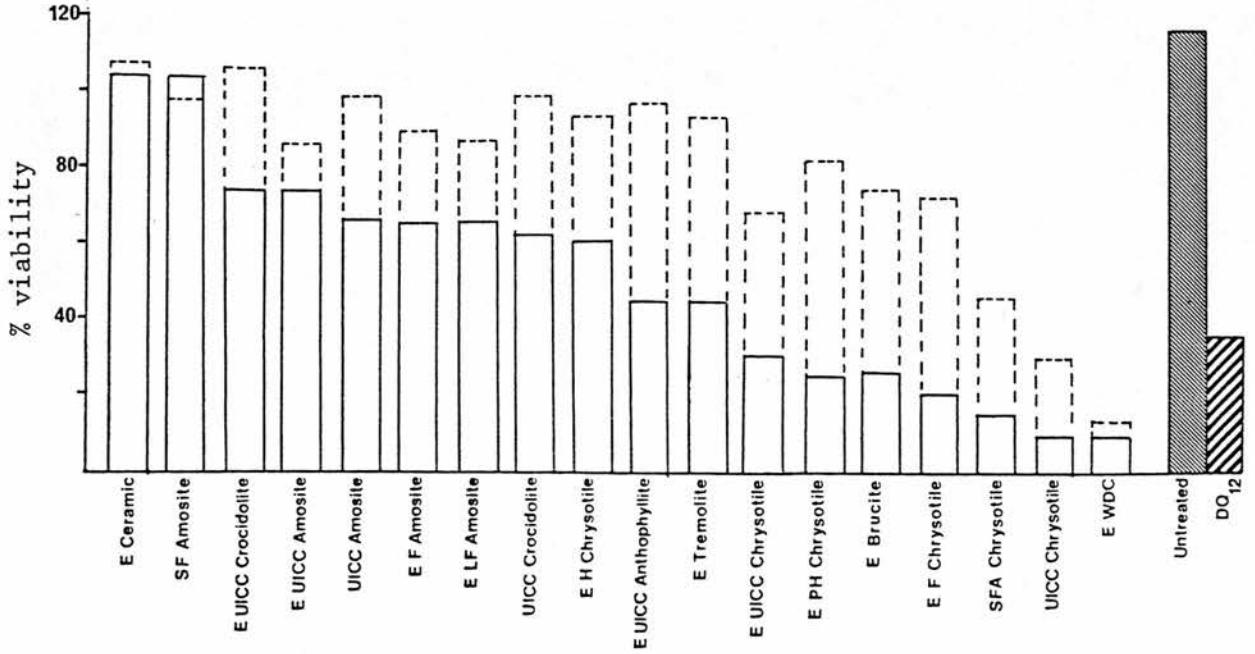

Figure 4.11 Percentage Viability 48 Hrs Following Treatment of $P388D_1$ Cells With Group 1 Dusts.

observed at 48 hrs rather than at 24 hrs. In general, a greater difference was seen between the two concentrations at 48 hrs than at the 24 hrs time point, and greater differences between individual dust samples could be seen at the 48 hrs time point. Whilst the order of cytotoxicity was not exactly the same for the two concentrations of dust at the 24 and 48 hrs time points, the overall trends were similar, thereby justifying the sequential order in which the dusts had been placed on the histogram.

When considering the cytotoxic effect of the various fibre samples, with respect to alterations in the viability of the $P388D_1$ cells, the serpentine samples, with the exception of E H chrysotile, proved to be more cytotoxic than the amphiboles, although the difference between the least cytotoxic serpentine and the most cytotoxic amphibole was not statistically significant at either of the two dust concentrations or time points. E ceramic fibre proved the least cytotoxic of all of the Group 1 fibrous samples examined, although this sample did not prove to be significantly different from SF amosite. E WDC consistently showed the greatest level of cytotoxicity, although the values obtained were statistically significantly different from the UICC chrysotile samples at the 10 μg/ml concentration only ($p<0.05$ for E WDC and UICC chrysotile at 24 and 48 hrs). E tremolite and E anthophyllite showed a similar degree of cytotoxicity towards $P388D_1$ cells, according to this assay system, and were more cytotoxic than the remainder of the amphibole samples ($p<0.05$ between UICC crocidolite and E tremolite at 50 μg/ml for 48 hrs). The E brucite ($Mg(OH)_2$) sample consistently proved more cytotoxic than the amphiboles, and displayed a similar degree of cytotoxicity towards $P388D_1$ cells as the E F chrysotile samples at all concentrations and time points ($p>0.1$). SF amosite proved less cytotoxic than the E LF amosite parent sample ($p<0.001$ between SF amosite and E LF amosite at 50 μg/ml for 48 hrs); E H chrysotile also proved less cytotoxic than the E PH chrysotile parent sample ($p<0.02$ between E H and E PH chrysotile at 50 μg/ml for 48 hrs).

4.3.3.2 Lactate Production.

The lactate production by the $P388D_1$ cells at 24 and 48 hrs following treatment with the Group 1 dust samples is shown in Table 4.6 and Figures 4.12 and 4.13. It was not possible to find a general trend between cytotoxic potential and lactate production, as a 50 μg/ml concentration of fibres did not necessarily consistently induce a greater or lower production of lactate than a 10 μg/ml concentration; in addition no relationship could be seen between the time of duration of treatment and the lactate production. It was observed that the majority of the dust samples tended to induce a similar lactate production from $P388D_1$ cells to the TiO_2 control treatment; those samples that appeared to induce a production of greater than 100% also had large standard deviations around the means for the variables, thereby removing the statistical significance of the result. The fibrous sample of E brucite, at a concentration of 50 μg/ml, induced the most significant decrease in lactate production at 48 hrs ($p<0.01$ between E brucite and E PH chrysotile at 50 μg/ml for 48 hrs).

4.3.3.3 LDH Levels.

The measured intracellular levels of the enzyme LDH, 24 and 48 hrs following the treatment of $P388D_1$ cells with Group 1 dusts are shown in Table 4.7 and Figures 4.14 and 4.15. It can be seen that a concentration of 50 μg/ml of dust consistently caused a greater loss of intracellular LDH than the 10 μg/ml concentration at 24 and 48 hrs; in addition, a greater loss of LDH was observed after 48 hrs than after 24 hrs from those cells treated with the more cytotoxic dusts (Figures 4.14 and 4.15). No statistically significant increase in intracellular LDH was observed following treatment with any of the dust samples. When considering the cytotoxic potential of those dust samples with differing physicochemical properties, the serpentines, at both concentrations of dust, did not necessarily cause a greater loss of intracellular LDH than the amphiboles at both time points. E WDC, however, consistently proved to be the most cytotoxic of all the samples examined, although the results for E WDC did not prove statistically significantly different from those for UICC chrysotile. Of the amphibole group, E UICC anthophyllite caused the greatest loss of

Table 4.6 Lactate Production for P388D_1 Cells Following Treatment With Group 1 Dusts.

Treatment	24 Hrs		48 Hrs	
	10 µg/ml	50 µg/ml	10 µg/ml	50 µg/ml
E Ceramic	90.8 ± 13.9	118.5 ± 19.6	116.8 ± 23.5	110.2 ± 9.6
UICC Crocidolite	112.7 ± 29.4	107.6 ± 36.1	95.6 ± 12.1	97.3 ± 23.8
E UICC Crocidolite	103.9 ± 5.1	92.6 ± 22.9	94.3 ± 6.1	96.3 ± 9.9
UICC Amosite	107.1 ± 24.4	124.8 ± 35.9	102.2 ± 7.5	99.7 ± 23.7
E UICC Amosite	122.6 ± 26.3	106.3 ± 16.6	118.6 ± 16.2	99.7 ± 13.0
E F Amosite	109.2 ± 2.6	94.0 ± 14.1	113.2 ± 25.0	94.5 ± 20.9
SF Amosite	153.5 ± 62.8	96.8 ± 8.6	136.1 ± 30.5	102.3 ± 4.5
E LF Amosite	94.8 ± 15.7	134.4 ± 50.2	99.2 ± 3.1	110.7 ± 26.3
E Tremolite	79.3 ± 28.3	75.1 ± 26.5	73.5 ± 32.9	52.8 ± 14.7
E UICC Anthophyllite	95.3 ± 61.2	126.2 ± 49.4	83.0 ± 62.2	111.2 ± 12.8
E Brucite	63.7 ± 26.1	46.2 ± 8.2	71.7 ± 25.9	32.3 ± 8.3
UICC Chrysotile	107.4 ± 17.0	98.2 ± 7.8	156.2 ± 154.4	127.5 ± 118.4
E UICC Chrysotile	110.1 ± 9.6	126.1 ± 11.6	103.8 ± 7.2	82.3 ± 8.1
SFA Chrysotile	110.4 ± 21.0	109.4 ± 34.4	103.1 ± 11.4	114.8 ± 45.5
E F Chrysotile	183.7 ± 153.7	162.5 ± 110.9	110.5 ± 15.0	80.5 ± 8.5
E PH Chrysotile	103.8 ± 3.9	104.3 ± 11.0	106.9 ± 7.0	77.5 ± 11.5
E H Chrysotile	125.3 ± 39.2	117.6 ± 23.0	127.6 ± 30.0	107.7 ± 11.6
E WDC	140.2 ± 75.5	163.4 ± 73.7	83.9 ± 39.8	88.3 ± 28.0
Untreated Control	113.9 ± 47.8		106.2 ± 30.8	
DQ_{12}	96.8 ± 24.8		75.4 ± 17.5	

Results are expressed as a percentage of the value obtained for the TiO_2 control. Each figure is a mean of at least 3 experiments ± SD.

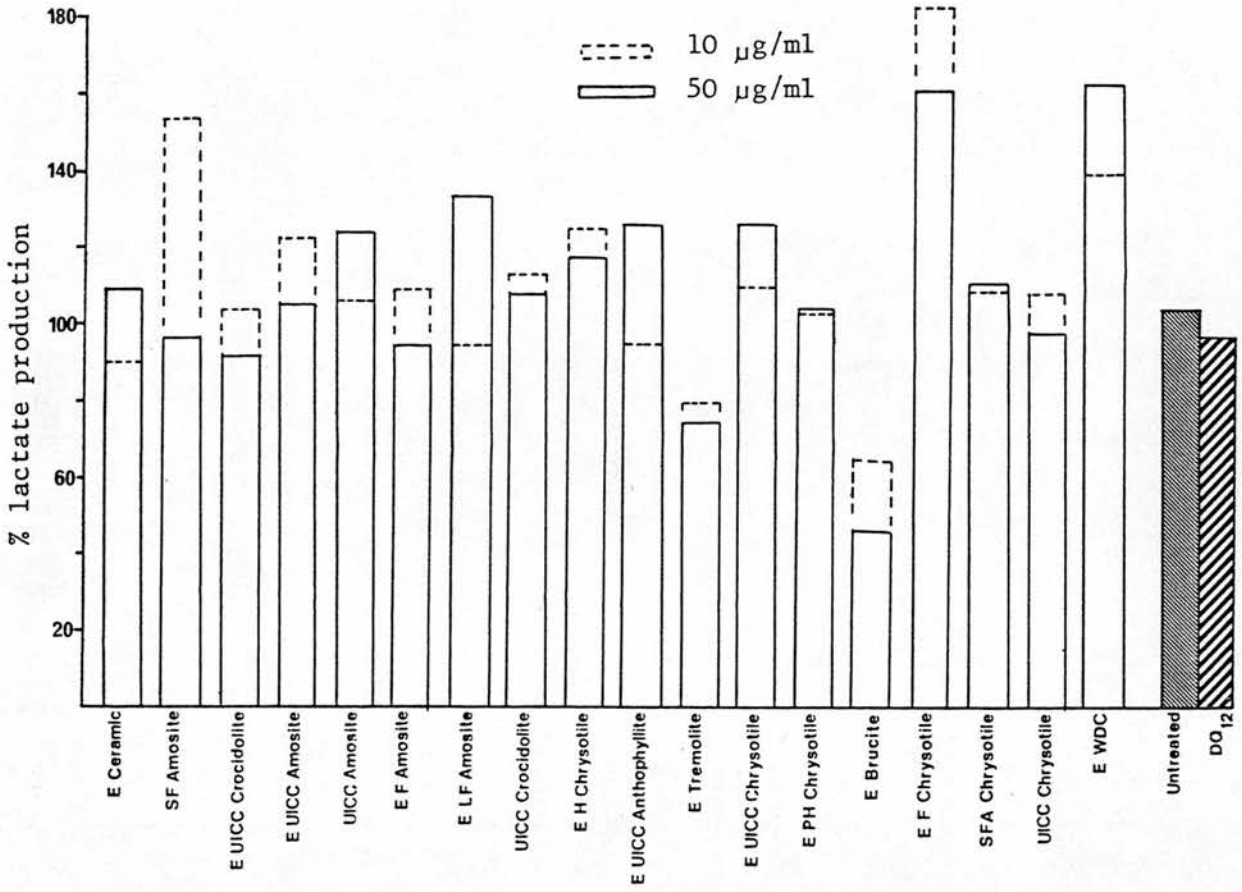

Figure 4.12 Lactate Production 24 Hrs Following Treatment of P388D$_1$ Cells With Group 1 Dusts.

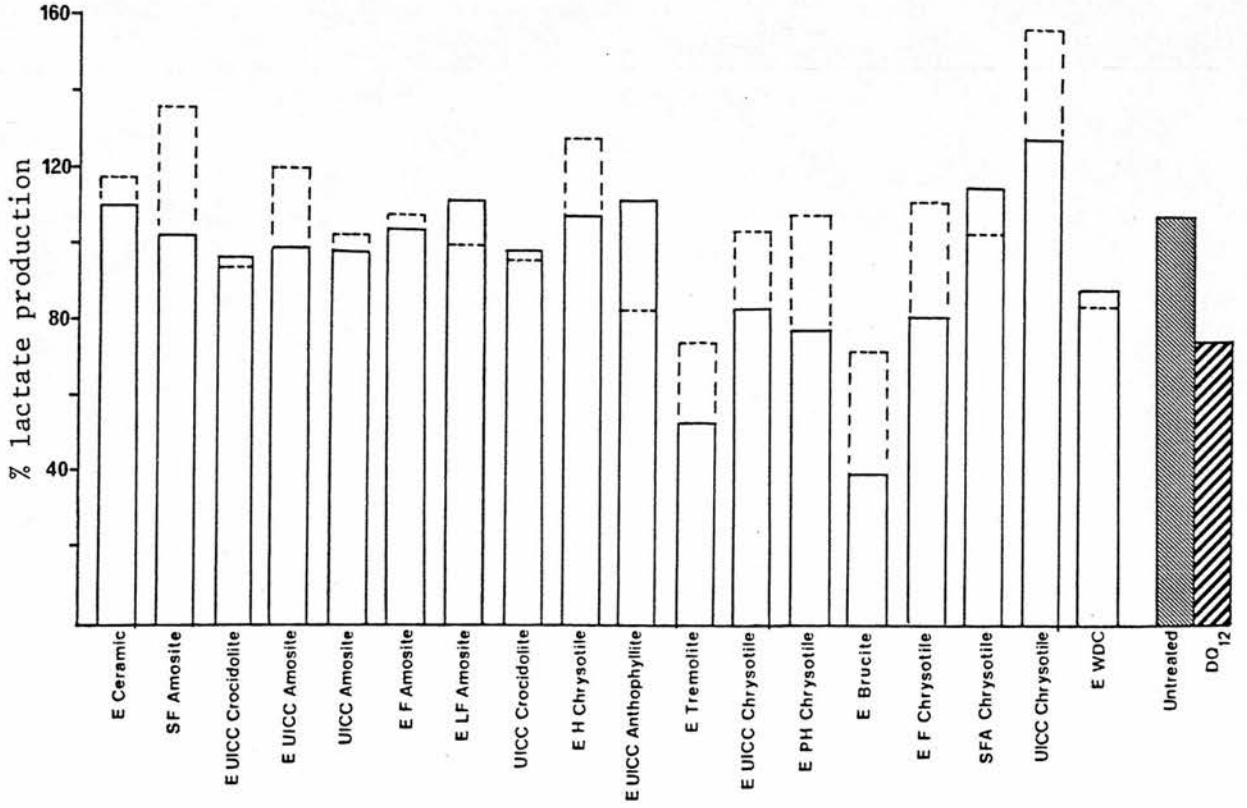

Figure 4.13 Lactate Production 48 Hrs Following Treatment of P388D$_1$ Cells With Group 1 Dusts.

Table 4.7 Intracellular LDH Levels Following Treatment with Group 1 Dusts.

Treatment	24 Hrs		48 Hrs	
	10 μg/ml	50 μg/ml	10 μg/ml	50 μg/ml
E Ceramic	95.3 ± 19.6	95.5 ± 20.3	109.6 ± 14.9	105.1 ± 13.3
UICC Crocidolite	105.3 ± 6.2	95.1 ± 5.5	99.8 ± 17.9	73.8 ± 14.0
E UICC Crocidolite	99.5 ± 9.6	97.2 ± 6.4	109.0 ± 10.0	96.5 ± 8.9
UICC Amosite	104.4 ± 5.9	97.1 ± 9.1	98.2 ± 5.2	75.9 ± 18.9
E UICC Amosite	107.9 ± 13.7	99.9 ± 16.0	119.3 ± 21.6	96.4 ± 19.5
E F Amosite	105.7 ± 10.8	98.7 ± 14.4	124.9 ± 32.7	95.2 ± 14.7
SF Amosite	102.7 ± 1.8	101.0 ± 3.4	104.5 ± 4.8	92.7 ± 13.6
E LF Amosite	98.2 ± 2.7	90.4 ± 5.2	98.5 ± 4.1	75.8 ± 4.7
E Tremolite	97.5 ± 0.8	89.2 ± 10.7	108.6 ± 12.6	60.7 ± 2.7
E UICC Anthophyllite	99.9 ± 3.6	82.8 ± 1.0	95.9 ± 23.0	46.9 ± 17.6
E Brucite	89.8 ± 17.8	57.1 ± 21.9	95.9 ± 2.6	49.0 ± 16.5
UICC Chrysotile	88.3 ± 12.6	55.8 ± 13.6	69.0 ± 29.1	46.9 ± 27.0
E UICC Chrysotile	88.4 ± 16.0	69.7 ± 13.8	87.3 ± 7.4	47.7 ± 12.8
SFA Chrysotile	101.5 ± 22.4	83.8 ± 15.0	86.7 ± 14.7	49.7 ± 17.8
E F Chrysotile	88.4 ± 14.7	64.2 ± 11.4	84.8 ± 15.1	42.7 ± 13.3
E PH Chrysotile	100.5 ± 17.3	78.3 ± 14.1	97.6 ± 9.6	51.2 ± 17.6
E H Chrysotile	107.5 ± 3.3	99.3 ± 3.0	115.3 ± 4.4	89.6 ± 10.3
E WDC	56.2 ± 23.0	43.6 ± 20.2	30.5 ± 16.3	22.9 ± 11.6
Untreated Control	105.4 ± 9.4		112.4 ± 16.0	
DQ_{12}	65.7 ± 17.3		51.7 ± 12.9	

Results are expressed as a percentage of the TiO_2 value.

Each figure is a mean of at least 3 experiments ± SD.

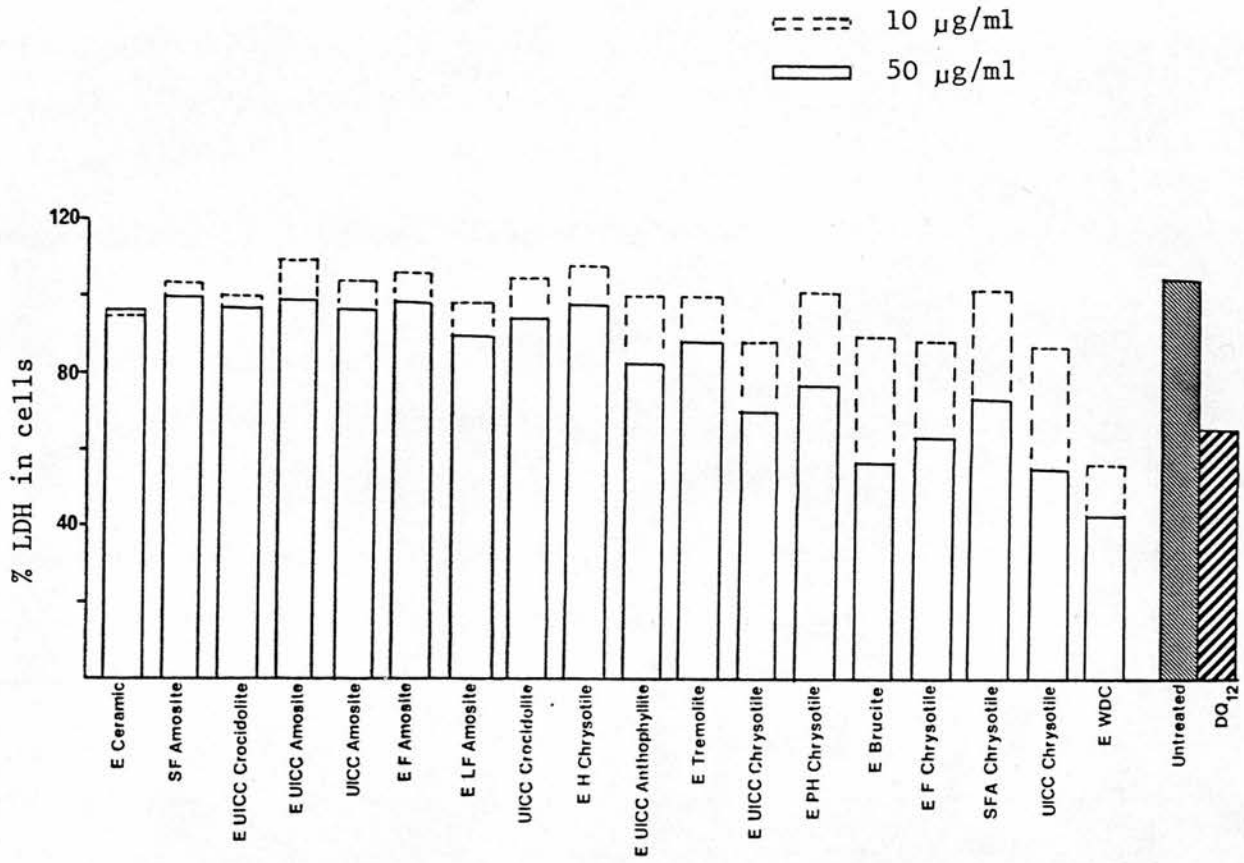

Figure 4.14 Intracellular LDH Levels 24 Hrs Following Treatment of $P388D_1$ Cells With Group 1 Dusts.

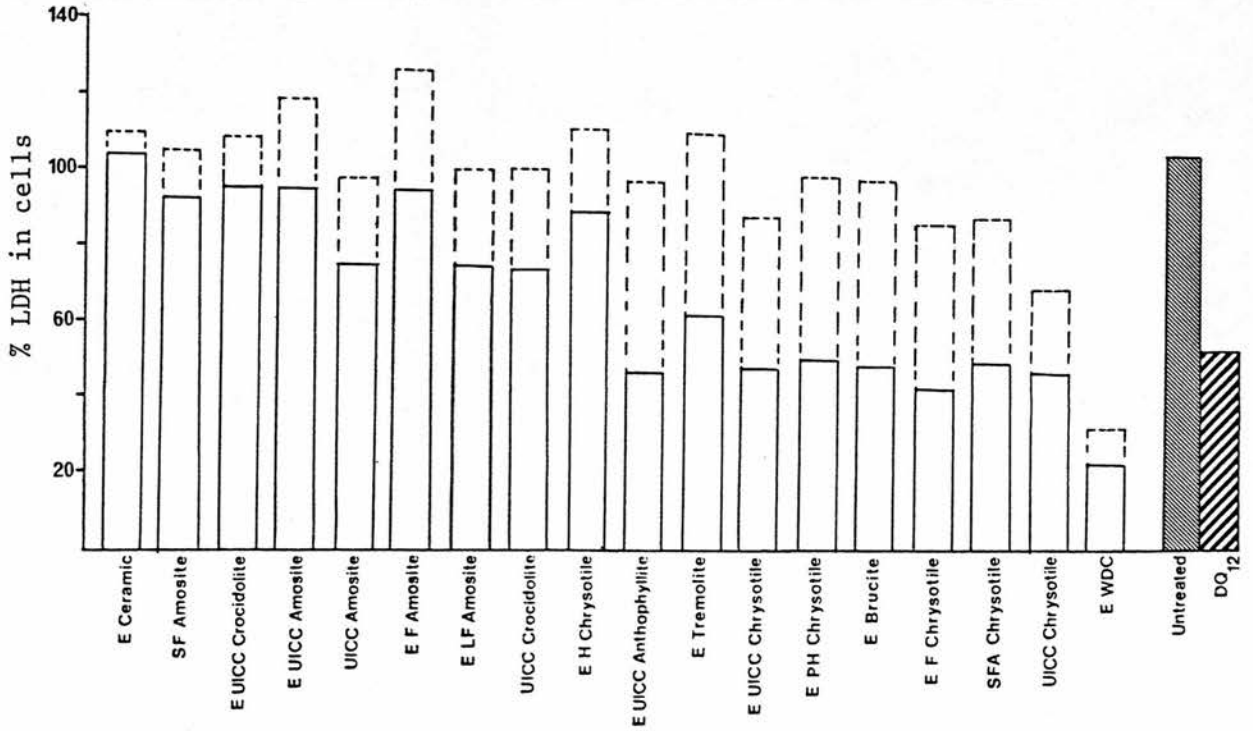

Figure 4.15 Intracellular LDH Levels 48 Hrs Following Treatment of $P388D_1$ Cells With Group 1 Dusts.

intracellular LDH, although the results for this dust were not significantly different from those for E tremolite. E ceramic fibre proved to be one of the least active fibrous samples, and E brucite caused a similar decrease of intracellular LDH to the E F chrysotile sample.

Elutriation of both UICC crocidolite and amosite resulted in a reduction in loss of intracellular LDH at the 50 μg/ml concentration for 48 hrs; although the levels for E UICC crocidolite and UICC crocidolite were not significantly different, treatment with the two amosite samples produced a statistically significant difference at 50 μg/ml for 48 hrs ($p<0.05$); this type of result was not so marked for the UICC and E UICC chrysotile samples. It was also observed that the SF amosite caused a smaller loss of intracellular enzyme than the E LF parent, although the results were not significantly different, and E H chrysotile also proved less cytotoxic than the E PH chrysotile parent sample ($p<0.05$ between E H and E PH chrysotile at 50 μg/ml for 48 hrs).

The total levels of LDH per plate of $P388D_1$ cells treated with Group 1 fibrous samples is shown in Table 4.8 and Figures 4.16 and 4.17. The treatment of $P388D_1$ cells with any of the fibrous samples resulted in a value for the total LDH level that was never significantly different from the TiO_2 control. The values for the levels of LDH measured in the medium (percentage of LDH in medium and percentage of LDH released into the medium, expressed as a percentage of the TiO_2 control result) are shown in Tables 4.9 and 4.10. The standard deviations around the mean figure for each value is large, and this situation has occurred because of the small quantity of LDH released by the TiO_2 treated control cells (see Section 4.3.2.4); because of this finding it was not possible to present a meaningful description of the data for Tables 4.9 and 4.10.

4.3.3.4 Glucosaminidase Levels.

The levels of intracellular glucosaminidase following the treatment of $P388D_1$ cells with Group 1 dusts are shown in Table 4.11 and Figures 4.18 and 4.19. Before these results are discussed it should be noted that levels of intracellular glucosaminidase of greater than 100% in value may be observed for some of the test samples (Figures 4.18

Table 4.8 Total Levels of LDH Per Plate Following Treatment With Group 1 Dusts.

Treatment	24 Hrs		48 Hrs	
	10 μg/ml	50 μg/ml	10 μg/ml	50 μg/ml
E Ceramic	97.4 ± 15.1	92.4 ± 11.8	107.9 ± 3.8	134.7 ± 74.7
UICC Crocidolite	109.0 ± 10.6	102.7 ± 10.4	105.3 ± 9.5	100.6 ± 12.4
E UICC Crocidolite	111.9 ± 19.5	110.0 ± 19.9	108.0 ± 4.9	111.0 ± 11.5
UICC Amosite	111.6 ± 7.7	103.2 ± 8.9	108.5 ± 9.6	104.4 ± 12.4
E UICC Amosite	112.5 ± 4.4	100.0 ± 12.4	121.5 ± 18.3	113.5 ± 3.9
E F Amosite	111.6 ± 17.0	106.3 ± 16.2	161.3 ± 94.3	101.5 ± 6.7
SF Amosite	108.5 ± 6.1	107.3 ± 10.7	120.0 ± 10.0	107.4 ± 9.8
E LF Amosite	111.5 ± 12.4	103.4 ± 12.1	119.1 ± 5.2	121.3 ± 2.8
E Tremolite	101.5 ± 7.6	105.7 ± 4.6	129.8 ± 25.5	105.9 ± 4.5
E UICC Anthophyllite	110.2 ± 12.1	109.9 ± 10.3	112.1 ± 10.2	109.3 ± 3.0
E Brucite	109.8 ± 6.1	108.8 ± 14.0	111.1 ± 7.8	104.0 ± 9.6
UICC Chrysotile	104.2 ± 12.4	102.7 ± 7.4	105.2 ± 20.1	116.1 ± 23.0
E UICC Chrysotile	100.0 ± 6.3	104.2 ± 2.6	100.6 ± 3.2	105.3 ± 13.6
SFA Chrysotile	107.5 ± 7.7	103.5 ± 5.6	103.7 ± 2.4	105.4 ± 14.4
E F Chrysotile	100.5 ± 9.0	95.2 ± 10.5	96.5 ± 17.9	80.2 ± 22.1
E PH Chrysotile	102.6 ± 3.1	99.3 ± 13.8	101.2 ± 5.4	97.0 ± 15.5
E H Chrysotile	104.2 ± 11.2	91.8 ± 14.9	105.1 ± 15.0	99.8 ± 13.4
E WDC	100.6 ± 8.8	98.8 ± 3.9	98.5 ± 20.8	93.3 ± 10.7
Untreated Control	108.3 ± 13.1		110.2 ± 17.0	
DQ_{12}	100.8 ± 11.6		101.1 ± 10.8	

Results are expressed as a percentage of the TiO_2 value.

Each figure is a mean of at least 3 experiments ± SD.

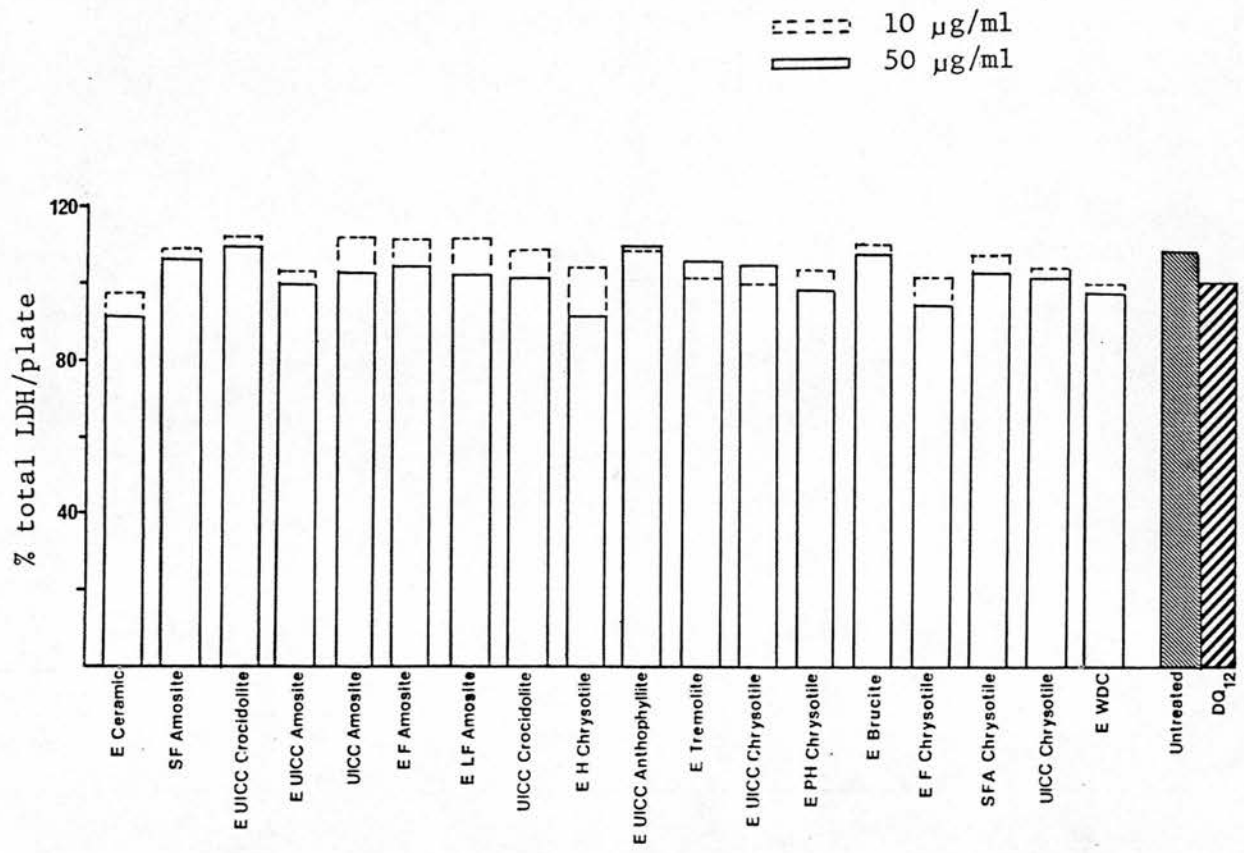

Figure 4.16 Total Levels of LDH/Plate 24 Hrs Following Treatment of $P388D_1$ Cells With Group 1 Dusts.

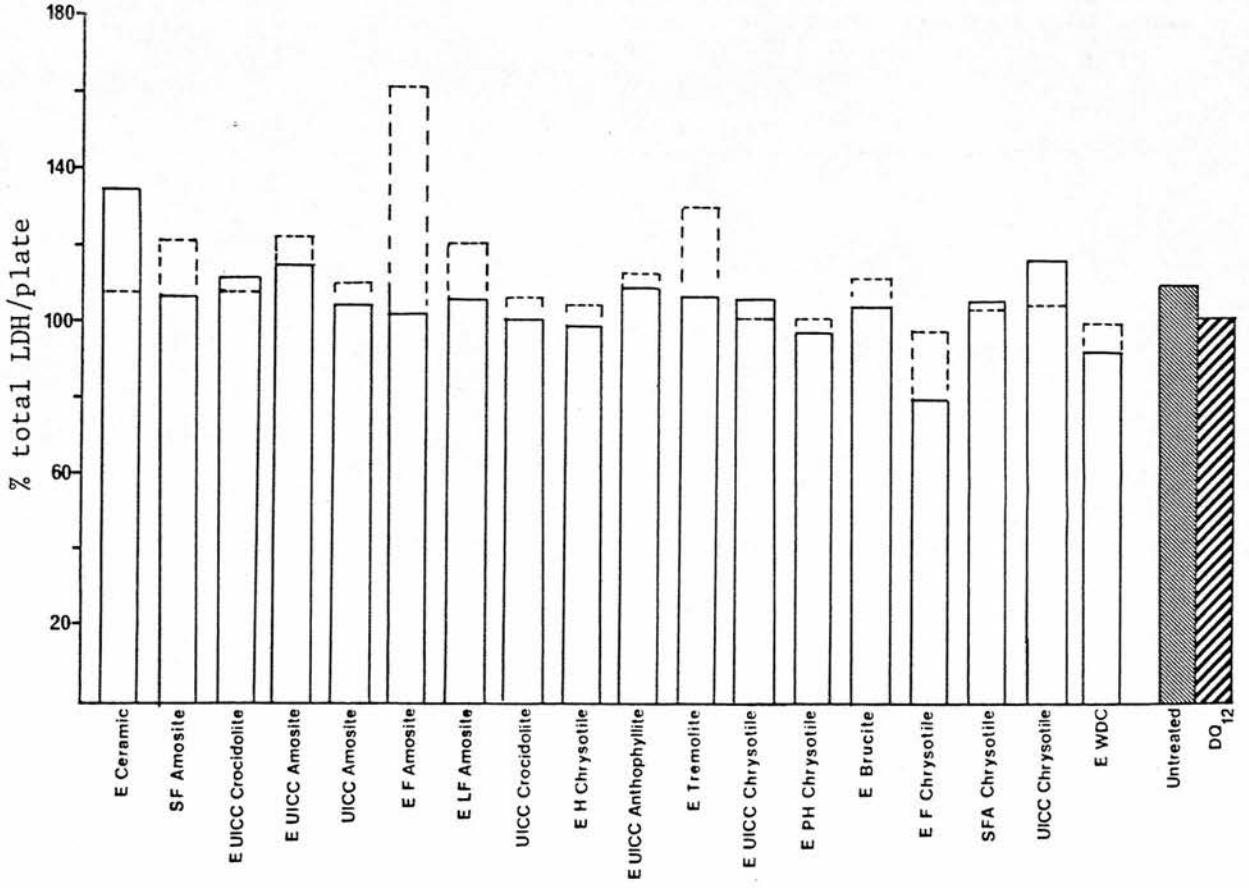

Figure 4.17 Total Levels of LDH/Plate 48 Hrs Following Treatment of $P388D_1$ Cells With Group 1 Dusts.

Table 4.9 LDH Levels In Medium Following Treatment With Group 1 Dusts.

Treatment	24 Hrs		48 Hrs	
	10 µg/ml	50 µg/ml	10 µg/ml	50 µg/ml
E Ceramic	127 ± 52	101 ± 37	117 ± 21	262 ± 323
UICC Crocidolite	169 ± 91	157 ± 81	169 ± 82	309 ± 216
E UICC Crocidolite	3026 ± 4207	1733 ± 2357	71 ± 0	1072 ± 1581
UICC Amosite	146 ± 21	144 ± 60	169 ± 43	263 ± 78
E UICC Amosite	161 ± 49	167 ± 132	305 ± 276	1119 ± 1387
E F Amosite	131 ± 32	145 ± 7	108 ± 11	473 ± 51
SF Amosite	152 ± 68	153 ± 63	203 ± 59	206 ± 26
E LF Amosite	144 ± 38	148 ± 53	231 ± 58	362 ± 28
E Tremolite	221 ± 211	552 ± 534	235 ± 200	376 ± 104
E UICC Anthophyllite	353 ± 292	512 ± 351	164 ± 116	394 ± 52
E Brucite	528 ± 527	1007 ± 937	153 ± 107	505 ± 100
UICC Chrysotile	204 ± 69	403 ± 227	230 ± 98	321 ± 119
E UICC Chrysotile	199 ± 112	356 ± 26	152 ± 35	294 ± 107
SFA Chrysotile	186 ± 103	257 ± 156	168 ± 51	288 ± 123
E F Chrysotile	256 ± 214	422 ± 281	144 ± 27	232 ± 86
E PH Chrysotile	108 ± 24	191 ± 62	122 ± 27	283 ± 102
E H Chrysotile	86 ± 52	113 ± 33	79 ± 48	151 ± 61
E WDC	300 ± 19	377 ± 239	378 ± 42	387 ± 76
Untreated Control	285 ± 690		133 ± 158	
DQ_{12}	1526 ± 2798		521 ± 509	

Results are expressed as a percentage of the TiO_2 value.

Each figure is a mean of at least 3 experiments ± SD.

Table 4.10 Overall Percentage of LDH Released Into The Medium Following Treatment With Group 1 Dusts.

Treatment	24 Hrs		48 Hrs	
	10 µg/ml	50 µg/ml	10 µg/ml	50 µg/ml
E Ceramic	133 ± 49	118 ± 60	109 ± 22	151 ± 109
UICC Crocidolite	120 ± 45	158 ± 92	155 ± 75	295 ± 163
E UICC Crocidolite	62 ± 0	76 ± 0	35 ± 50	313 ± 301
UICC Amosite	130 ± 13	118 ± 82	124 ± 79	252 ± 79
E UICC Amosite	144 ± 46	158 ± 112	298 ± 286	157 ± 40
E F Amosite	118 ± 26	139 ± 23	100 ± 0	506 ± 557
SF Amosite	143 ± 56	144 ± 67	169 ± 35	190 ± 17
E LF Amosite	140 ± 33	154 ± 53	191 ± 37	299 ± 20
E Tremolite	228 ± 209	562 ± 549	183 ± 103	351 ± 79
E UICC Anthophyllite	329 ± 277	492 ± 363	138 ± 93	357 ± 35
E Brucite	545 ± 573	1009 ± 1012	238 ± 93	487 ± 120
UICC Chrysotile	192 ± 73	402 ± 255	255 ± 105	277 ± 116
E UICC Chrysotile	205 ± 120	340 ± 239	152 ± 35	278 ± 91
SFA Chrysotile	180 ± 116	254 ± 169	163 ± 51	276 ± 119
E F Chrysotile	264 ± 209	493 ± 385	147 ± 14	290 ± 95
E PH Chrysotile	104 ± 24	226 ± 60	124 ± 31	294 ± 113
E H Chrysotile	78 ± 43	108 ± 25	71 ± 38	149 ± 53
E WDC	1009 ± 1138	1445 ± 1798	394 ± 112	419 ± 118
Untreated Control	147 ± 179		115 ± 123	
DQ_{12}	408 ± 411		475 ± 406	

Results are expressed as a percentage of the TiO_2 control value.
Each figure is a mean of at least 3 experiments ± SD.

Table 4.11 Intracellular Glucosaminidase Levels Following Treatment With Group 1 Dusts.

Treatment	24 Hrs		48 Hrs	
	10 µg/ml	50 µg/ml	10 µg/ml	50 µg/ml
E Ceramic	125.5 ± 15.2	125.9 ± 16.7	144.7 ± 30.3	122.1 ± 48.7
UICC Crocidolite	138.7 ± 30.1	112.1 ± 13.4	144.0 ± 30.5	92.2 ± 14.2
E UICC Crocidolite	151.2 ± 19.4	119.7 ± 18.6	163.6 ± 13.8	105.8 ± 15.7
UICC Amosite	121.5 ± 16.8	106.8 ± 16.5	136.6 ± 26.3	97.5 ± 29.5
E UICC Amosite	132.6 ± 7.2	116.2 ± 12.6	170.8 ± 49.6	127.9 ± 27.7
E F Amosite	131.9 ± 0.9	110.7 ± 4.4	175.0 ± 56.8	126.9 ± 24.1
SF Amosite	138.3 ± 8.5	121.2 ± 7.3	160.9 ± 15.0	127.4 ± 14.4
E LF Amosite	132.2 ± 15.6	102.3 ± 0.2	143.5 ± 14.4	94.1 ± 7.0
E Tremolite	173.4 ± 43.4	120.4 ± 38.0	243.9 ± 107.7	89.0 ± 3.4
E UICC Anthophyllite	181.6 ± 44.8	128.9 ± 26.9	250.3 ± 101.2	92.4 ± 27.0
E Brucite	136.5 ± 52.3	62.0 ± 21.8	184.3 ± 94.2	48.3 ± 8.0
UICC Chrysotile	80.4 ± 22.4	79.7 ± 11.2	56.9 ± 12.7	22.0 ± 14.8
E UICC Chrysotile	81.1 ± 11.8	55.1 ± 16.9	66.6 ± 7.6	35.4 ± 13.6
SFA Chrysotile	99.2 ± 24.0	77.8 ± 48.3	108.7 ± 45.6	40.4 ± 10.6
E F Chrysotile	100.4 ± 43.1	78.2 ± 67.8	79.2 ± 23.8	27.7 ± 11.9
E PH Chrysotile	106.7 ± 3.7	55.8 ± 13.7	101.2 ± 10.8	35.1 ± 6.1
E H Chrysotile	144.1 ± 14.5	111.5 ± 12.5	136.8 ± 24.7	81.3 ± 6.4
E WDC	49.5 ± 20.8	37.3 ± 16.7	38.7 ± 13.7	36.0 ± 16.8
Untreated control	141.8 ± 40.2		172.2 ± 65.3	
DQ_{12}	92.6 ± 32.8		72.5 ± 27.8	

Results are expressed as a percentage of the TiO_2 control value.
Each figure is a mean of at least 3 experiments ± SD.

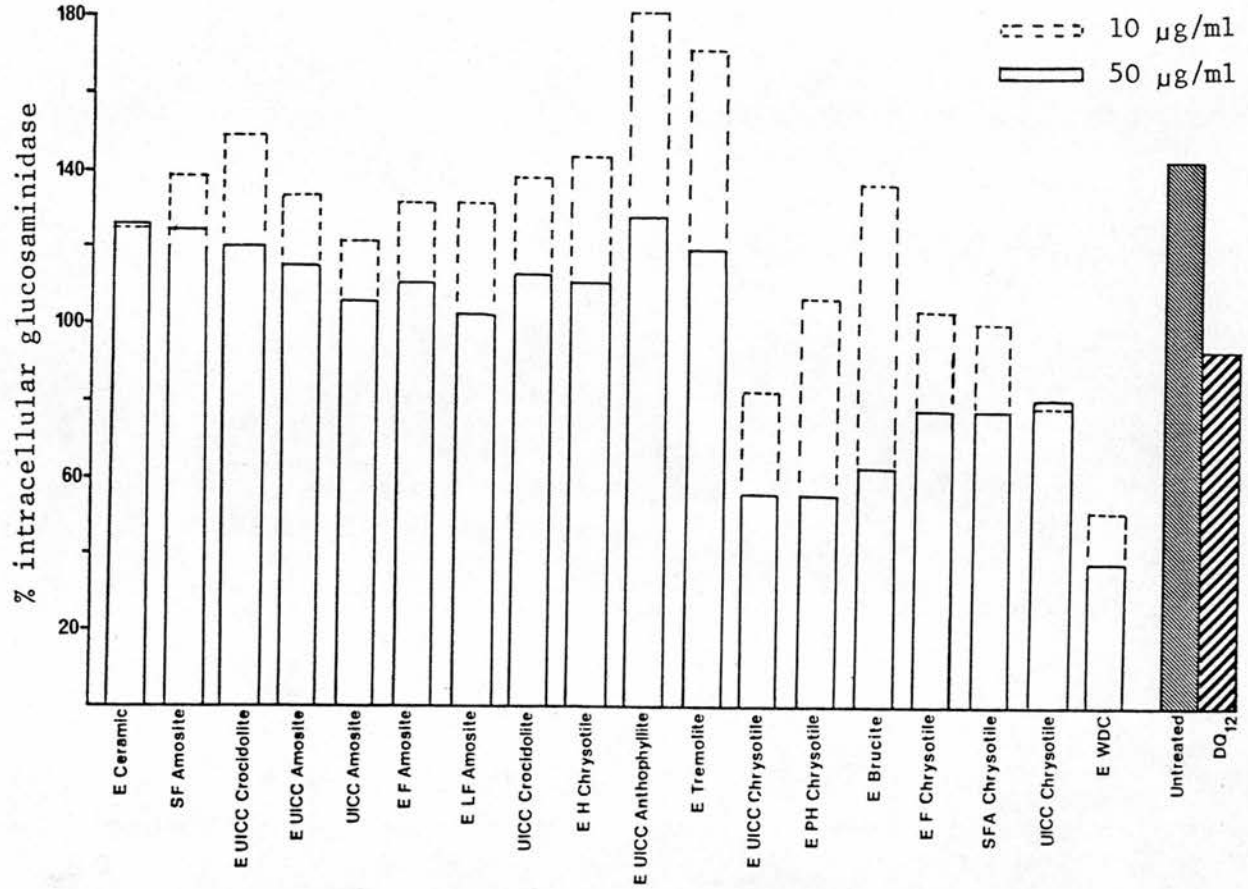

Figure 4.18 Intracellular Glucosaminidase Levels 24 Hrs Following Treatment of $P388D_1$ Cells With Group 1 Dusts.

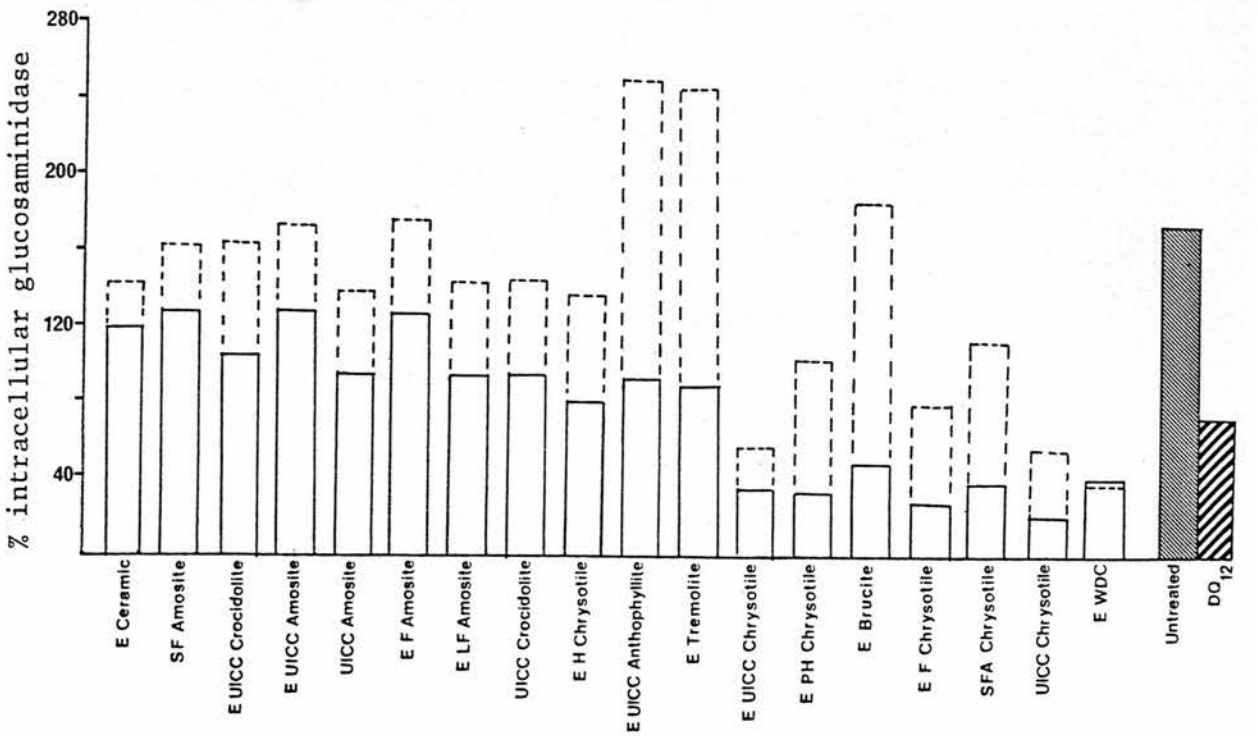

Figure 4.19 Intracellular Glucosaminidase Levels 48 Hrs Following Treatment of $P388D_1$ Cells With Group 1 Dusts.

and 4.19); this situation has occurred because of the large loss of intracellular glucosaminidase induced following treatment of the control cells with TiO_2 (see Section 4.3.2.5). It can be seen that the 50 μg/ml concentration of dust consistently caused a greater loss of intracellular glucosaminidase than the 10 μg/ml concentration at both time points (Figures 4.18 and 4.19); a greater loss of enzyme was observed at 48 hrs than at 24 hrs for the 10 μg/ml concentration, but this did not always occur for the 50 μg/ml concentration. With regard to the ability of fibres with different physicochemical properties to induce a loss of intracellular glucosaminidase the serpentines, with the exeption of E H chrysotile, caused a greater loss of enzyme than the amphiboles ($p<0.05$ between UICC chrysotile and E LF amosite at 50 μg/ml for 24 hrs, between SFA chrysotile and UICC amosite at 10 μg/ml for 48 hrs, and SFA chrysotile and E tremolite at 50 μg/ml for 48 hrs). The E WDC induced the greatest loss of enzyme at 10 and 50 μg/ml at 24 and 48 hrs. Elutriation of the UICC asbestos samples resulted in a reduction in the loss of intracellular glucosaminidase, although this reduction was not significant according to conventional analysis. E LF amosite induced a greater loss of intracellular glucosaminidase than the SF amosite sample ($p<0.05$ between E LF and SF amosite at 50 μg/ml for 48 hrs) and E PH chrysotile also induced a greater loss than E H chrysotile ($p<0.001$ between E PH and E H chrysotile at 50 μg/ml for 48 hrs). There was no evidence to suggest that a stimulation of intracellular enzyme had occurred; large values were obtained for E anthophyllite and E tremolite, but these were not significant because of the large standard deviations around the means for these values, and these large standard deviations occurred because one of the set of experimental results used to obtain these figures was inexplicably large.

The total levels of glucosaminidase available per plate of $P388D_1$ cells following treatment with Group 1 dusts is shown in Table 4.12 and Figures 4.20 and 4.21. It should be noted that the levels of enzyme for each dust treatment rarely achieved 100% of the TiO_2 control value. In general it can be seen that those dusts of a lower cytotoxic potential (as assessed by viability) showed a level of total glucosaminidase which was similar or higher in value than the untreated control level, whereas those dusts of a greater cytotoxic potential

Table 4.12 Total Levels of Glucosaminidase Per Plate Following Treatment With Group 1 Dusts.

Treatment	24 Hrs		48 Hrs	
	10 µg/ml	50 µg/ml	10 µg/ml	50 µg/ml
E Ceramic	114.8 ± 7.9	112.1 ± 6.6	125.4 ± 10.0	108.8 ± 26.2
UICC Crocidolite	133.0 ± 19.4	110.4 ± 11.8	147.2 ± 67.8	91.4 ± 18.0
E UICC Crocidolite	132.4 ± 12.7	117.1 ± 19.5	129.4 ± 10.2	102.4 ± 12.5
UICC Amosite	121.7 ± 13.7	110.5 ± 14.3	116.9 ± 16.4	95.1 ± 20.8
E UICC Amosite	131.0 ± 21.5	118.6 ± 18.5	139.7 ± 38.9	113.0 ± 32.1
E F Amosite	126.1 ± 13.6	111.4 ± 16.5	137.1 ± 33.9	105.6 ± 23.9
SF Amosite	128.3 ± 17.9	115.7 ± 11.5	128.9 ± 11.3	112.3 ± 8.0
E LF Amosite	132.4 ± 20.0	109.3 ± 14.5	129.3 ± 10.0	100.9 ± 12.8
E Tremolite	144.0 ± 17.9	116.4 ± 17.8	140.3 ± 20.9	82.7 ± 7.6
E UICC Anthophyllite	151.7 ± 15.0	130.9 ± 15.4	144.0 ± 17.8	91.7 ± 9.9
E Brucite	112.2 ± 31.4	62.2 ± 21.3	106.9 ± 21.5	39.4 ± 6.5
UICC Chrysotile	86.8 ± 18.1	59.6 ± 11.1	75.3 ± 14.7	55.1 ± 10.1
E UICC Chrysotile	87.8 ± 7.0	73.4 ± 13.1	83.4 ± 10.7	58.3 ± 10.7
SFA Chrysotile	96.6 ± 18.3	94.3 ± 8.7	99.5 ± 16.7	69.4 ± 16.5
E F Chrysotile	93.6 ± 24.2	97.5 ± 31.8	80.8 ± 21.4	49.4 ± 15.2
E PH Chrysotile	106.2 ± 13.3	76.3 ± 21.4	95.1 ± 2.0	50.4 ± 19.0
E H Chrysotile	134.4 ± 29.4	103.6 ± 7.6	121.9 ± 17.2	81.3 ± 8.3
E WDC	97.5 ± 13.7	76.4 ± 22.9	59.8 ± 24.9	54.2 ± 28.9
Untreated Control	129.3 ± 24.1		128.9 ± 27.2	
DQ_{12}	96.8 ± 18.9		97.1 ± 17.8	

Results are expressed as a percentage of the TiO_2 value.

Each figure is a mean of at least 3 experiments ± SD.

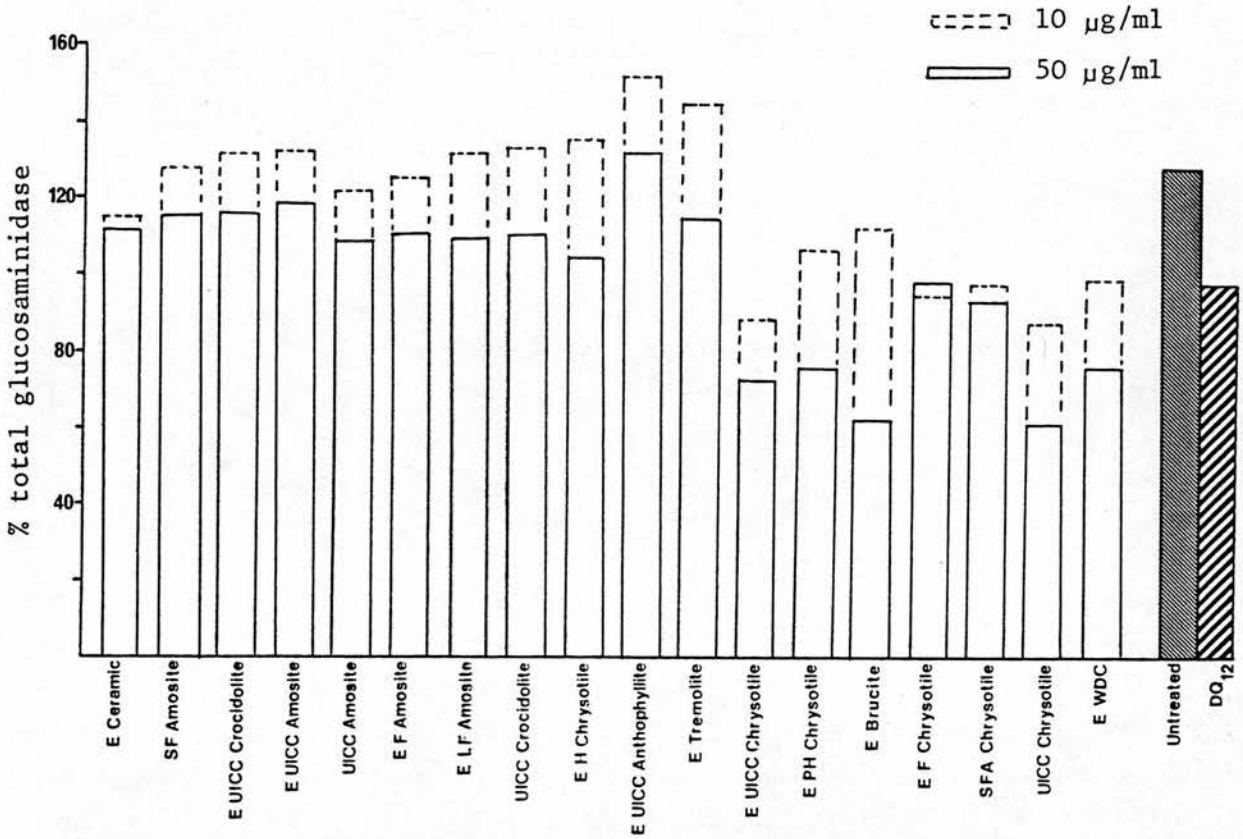

Figure 4.20 Total Levels of Glucosaminidase/Plate 24 Hrs Following Treatment of P388D$_1$ Cells With Group 1 Dusts.

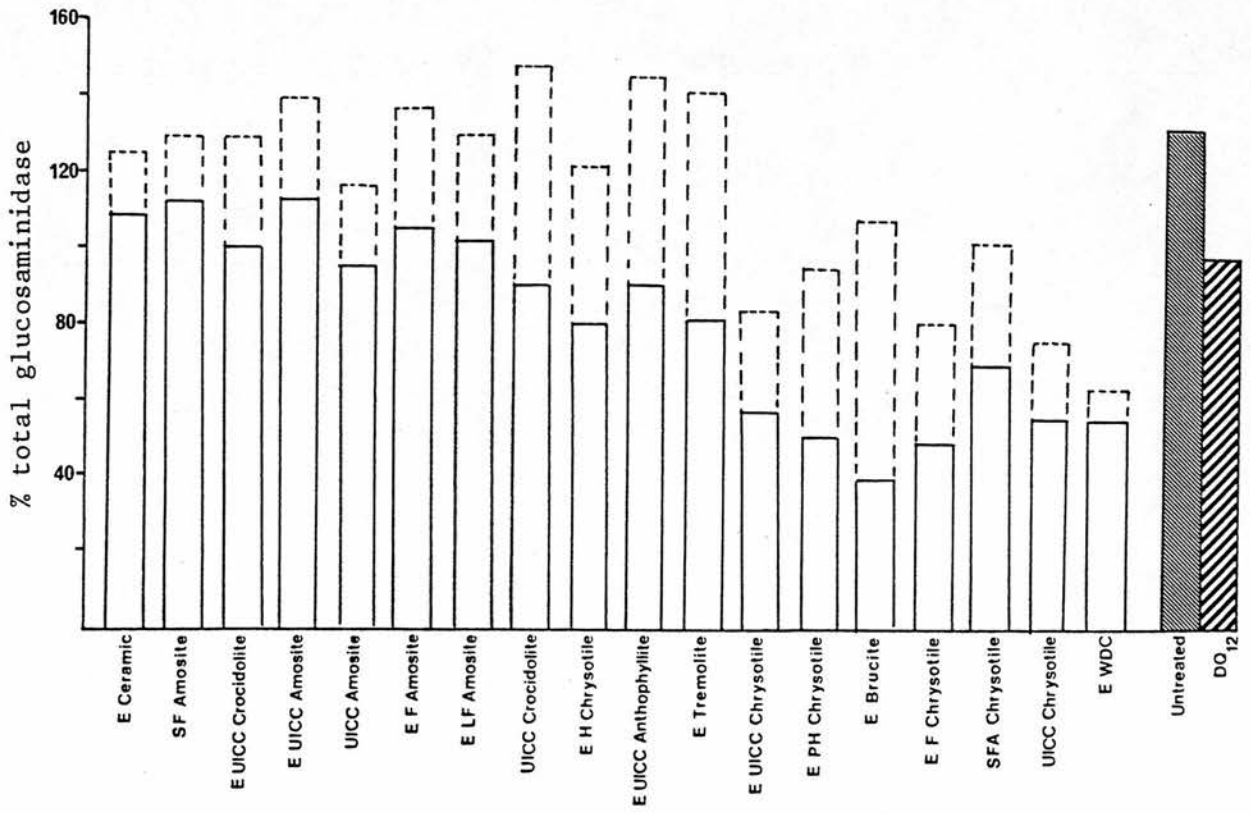

Figure 4.21 Total Levels of Glucosaminidase/Plate 48 Hrs Following Treatment of P388D$_1$ Cells With Group 1 Dusts.

possessed total enzyme levels that were similar to or less than the DQ_{12} value. A 50 μg/ml concentration of dust induced a greater reduction of the total enzyme level at both the 24 and 48 hrs time points than the 10 μg/ml concentration. The serpentines, with the exception of E H chrysotile, caused a greater reduction in the total glucosaminidase levels than the amphiboles ($p<0.05$ between E F chrysotile and E tremolite at 50 μg/ml for 48 hrs).

The percentage of glucosaminidase present in the culture medium 24 and 48 hrs following treatment with Group 1 dusts is shown in Table 4.13 and Figures 4.22 and 4.23. At the 24 hrs time point (Figure 4.23) it can be seen that treatment of the $P388D_1$ cells with 10 μg/ml of dust sometimes resulted in the presence of a greater quantity of enzyme in the medium than following treatment with 50 μg/ml. At 48 hrs, however, the 10 μg/ml concentration persistently resulted in greater enzyme levels than the 50 μg/ml concentration. In general, the level of enzyme present in the medium was lower at 48 hrs (Figure 4.23) than at 24 hrs (Figure 4.22).

The overall percentage of the total glucosaminidase per plate released into the medium following treatment of $P388D_1$ cells with Group 1 dusts is shown in Table 4.14 and Figures 4.24 and 4.25. A 50 μg/ml concentration of dust initiated the release of a greater percentage of enzyme than the 10 μg/ml concentration at the 24 hrs and 48 hrs time point; upon those occasions when the 10 μg/ml concentration appeared to induce a greater release than the 50 μg/ml concentration, eg for E ceramic, SF amosite and UICC crocidolite at 24 hrs, the difference between the two values for each dust was not statistically significant. In general, the values obtained for enzyme release at 24 hrs proved to be greater than the values obtained at 48 hrs. The more cytotoxic fibrous samples, as assessed by the ability of a dust to alter the cell viability, induced a greater level of enzyme release at 48 hrs than the less cytotoxic samples. The E LF amosite sample induced a greater release of glucosaminidase from $P388D_1$ cells than the SF amosite sample ($p<0.02$ between E LF and SF amosite at 50 μg/ml for 48 hrs) and E PH chrysotile also induced a greater release of the enzyme than E H chrysotile ($p<0.05$ between E PH and E H chrysotile at 50 μg/ml for 48 hrs). Treating the cells with

Table 4.13 Glucosaminidase Levels In Medium Following Treatment With Group 1 Dusts.

Treatment	24 Hrs		48 Hrs	
	10 µg/ml	50 µg/ml	10 µg/ml	50 µg/ml
E Ceramic	103.5 ± 10.2	95.3 ± 7.2	111.6 ± 14.1	105.8 ± 11.4
UICC Crocidolite	151.7 ± 93.9	121.5 ± 39.6	147.2 ± 98.3	89.4 ± 23.4
E UICC Crocidolite	103.1 ± 13.1	113.9 ± 28.5	102.3 ± 11.1	99.5 ± 11.0
UICC Amosite	143.6 ± 70.6	151.9 ± 90.8	105.1 ± 12.9	92.7 ± 16.8
E UICC Amosite	134.5 ± 42.4	124.7 ± 32.5	121.9 ± 38.9	103.2 ± 36.7
E F Amosite	124.2 ± 31.0	117.5 ± 38.2	116.3 ± 29.0	92.4 ± 27.5
SF Amosite	111.3 ± 46.3	103.0 ± 15.7	103.6 ± 15.6	100.0 ± 8.5
E LF Amosite	138.8 ± 38.2	84.9 ± 30.9	117.6 ± 13.1	106.5 ± 17.4
E Tremolite	124.6 ± 34.4	145.1 ± 77.0	104.5 ± 10.1	80.4 ± 13.2
E UICC Anthophyllite	135.2 ± 40.2	150.4 ± 35.1	106.4 ± 7.6	89.3 ± 3.0
E Brucite	87.7 ± 25.1	63.2 ± 19.3	81.2 ± 8.9	44.4 ± 3.3
UICC Chrysotile	107.6 ± 22.5	102.4 ± 58.2	83.2 ± 13.7	73.1 ± 21.2
E UICC Chrysotile	100.3 ± 14.4	105.5 ± 28.3	99.5 ± 20.2	80.3 ± 15.7
SFA Chrysotile	121.2 ± 27.4	116.0 ± 39.2	97.1 ± 7.8	78.3 ± 12.4
E F Chrysotile	93.4 ± 13.1	92.4 ± 2.7	82.0 ± 19.9	65.0 ± 11.9
E PH Chrysotile	107.1 ± 31.6	108.5 ± 41.2	88.7 ± 14.4	66.6 ± 31.2
E H Chrysotile	125.5 ± 54.4	94.9 ± 21.5	107.0 ± 11.7	80.6 ± 22.8
E WDC	114.9 ± 15.5	128.2 ± 48.6	73.8 ± 26.2	83.6 ± 30.9
Untreated Control	118.3 ± 29.9		108.7 ± 22.9	
DQ_{12}	122.2 ± 29.4		79.2 ± 17.9	

Results are expressed as a percentage of the TiO_2 control value.

Each figure is a mean of at least 3 experiments ± SD.

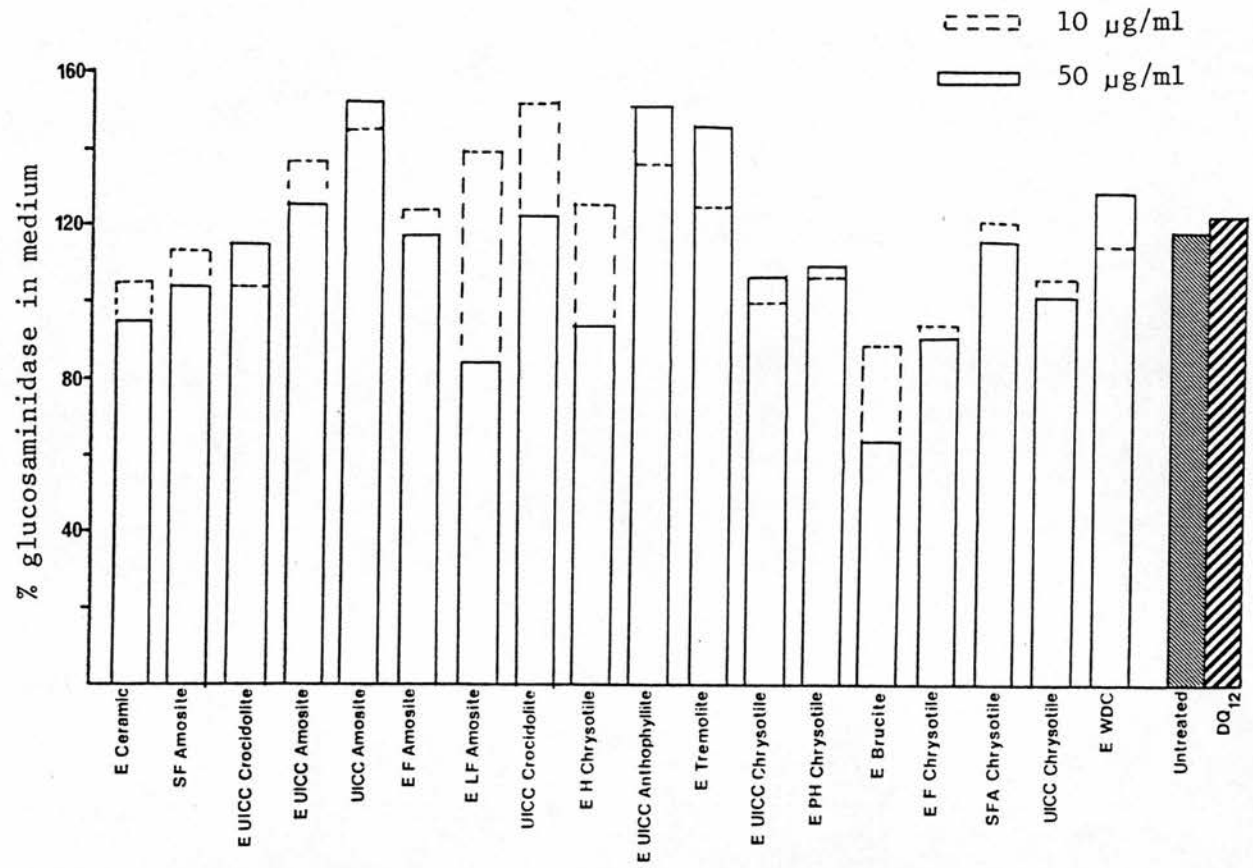

Figure 4.22 Glucosaminidase Levels in Medium 24 Hrs Following Treatment of $P388D_1$ Cells With Group 1 Dusts.

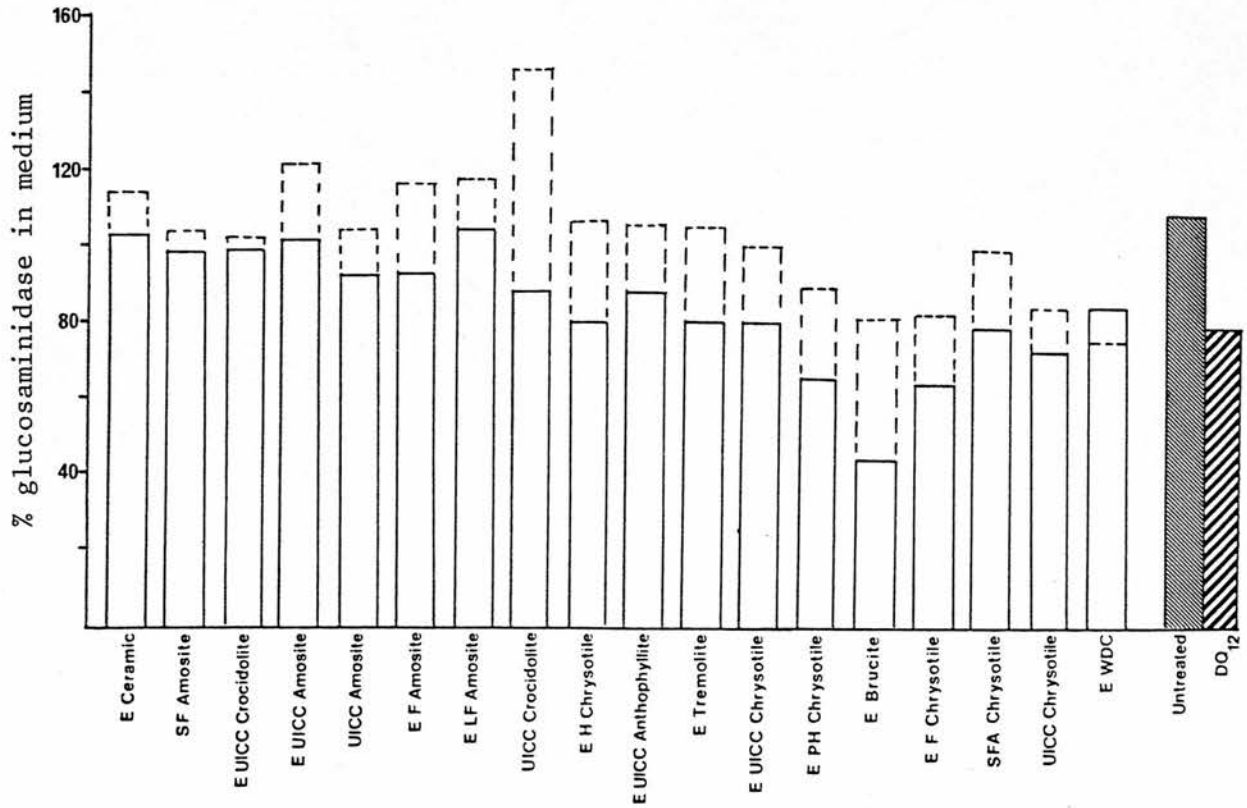

Figure 4.23 Glucosaminidase Levels in Medium 48 Hrs Following Treatment of $P388D_1$ Cells With Group 1 Dusts.

Table 4.14 Overall Percentage of Glucosaminidase Released Into the Medium Following Treatment With Group 1 Dusts.

Treatment	24 Hrs		48 Hrs	
	10 µg/ml	50 µg/ml	10 µg/ml	50 µg/ml
E Ceramic	90.5 ± 10.2	84.2 ± 6.7	89.7 ± 13.2	97.0 ± 25.8
UICC Crocidolite	116.4 ± 73.0	108.2 ± 31.3	95.9 ± 16.0	99.2 ± 6.9
E UICC Crocidolite	77.6 ± 2.4	97.8 ± 11.4	78.7 ± 2.8	97.4 ± 3.7
UICC Amosite	116.5 ± 48.5	137.3 ± 83.9	92.2 ± 7.6	100.6 ± 10.4
E UICC Amosite	101.4 ± 14.9	104.3 ± 10.3	86.4 ± 3.7	90.4 ± 5.9
E F Amosite	96.2 ± 13.6	100.3 ± 10.7	85.1 ± 2.6	86.8 ± 5.5
SF Amosite	91.1 ± 17.1	89.0 ± 5.3	80.1 ± 8.5	89.6 ± 6.4
E LF Amosite	104.2 ± 12.6	131.8 ± 54.4	92.0 ± 6.4	106.1 ± 4.0
E Tremolite	85.8 ± 21.6	125.5 ± 69.1	74.8 ± 7.0	96.8 ± 6.8
E UICC Anthophyllite	89.5 ± 27.4	115.6 ± 28.0	74.3 ± 5.3	98.0 ± 7.3
E Brucite	78.7 ± 0.4	91.8 ± 18.9	76.9 ± 8.5	97.5 ± 3.1
UICC Chrysotile	128.9 ± 32.8	181.8 ± 114.0	116.8 ± 12.7	148.3 ± 36.8
E UICC Chrysotile	128.3 ± 23.8	141.1 ± 12.9	119.6 ± 12.4	140.1 ± 10.3
SFA Chrysotile	120.9 ± 23.5	153.5 ± 66.6	165.2 ± 171.8	156.7 ± 67.3
E F Chrysotile	102.6 ± 11.2	132.4 ± 43.0	102.3 ± 4.0	135.7 ± 16.0
E PH Chrysotile	99.1 ± 15.6	119.1 ± 25.8	93.1 ± 12.8	132.8 ± 14.8
E H Chrysotile	90.8 ± 19.4	91.6 ± 16.7	88.7 ± 10.1	97.4 ± 16.1
E WDC	154.4 ± 46.5	169.3 ± 51.3	126.7 ± 15.5	134.5 ± 14.7
Untreated Control	90.8 ± 18.2		83.9 ± 9.4	
DQ_{12}	135.7 ± 31.2		104.1 ± 14.9	

Results are expressed as a percentage of the TiO_2 value.

Each figure is a mean of at least 3 experiments ± SD.

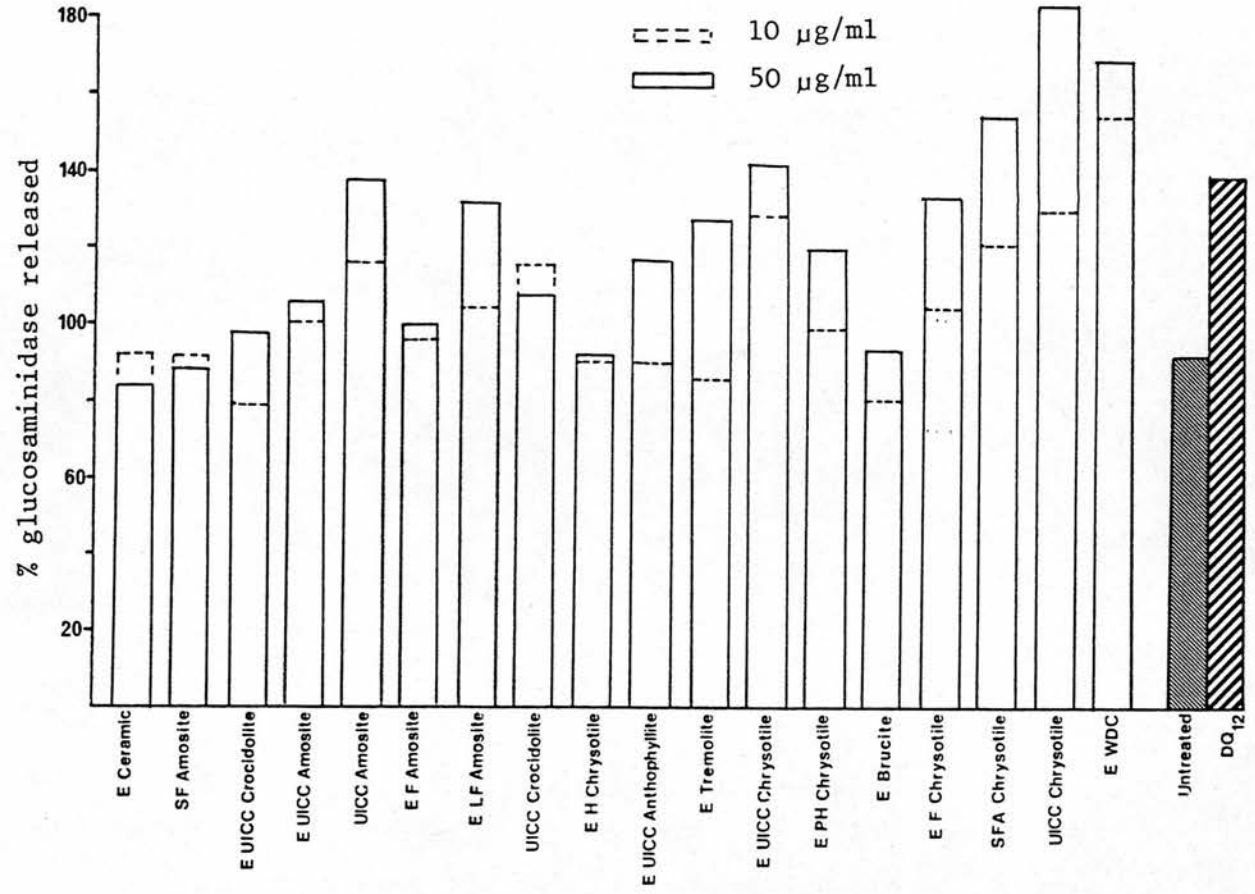

Figure 4.24 Overall Percentage of Glucosaminidase Released Into Medium Following Treatment of $P388D_1$ Cells With Group 1 Dusts.

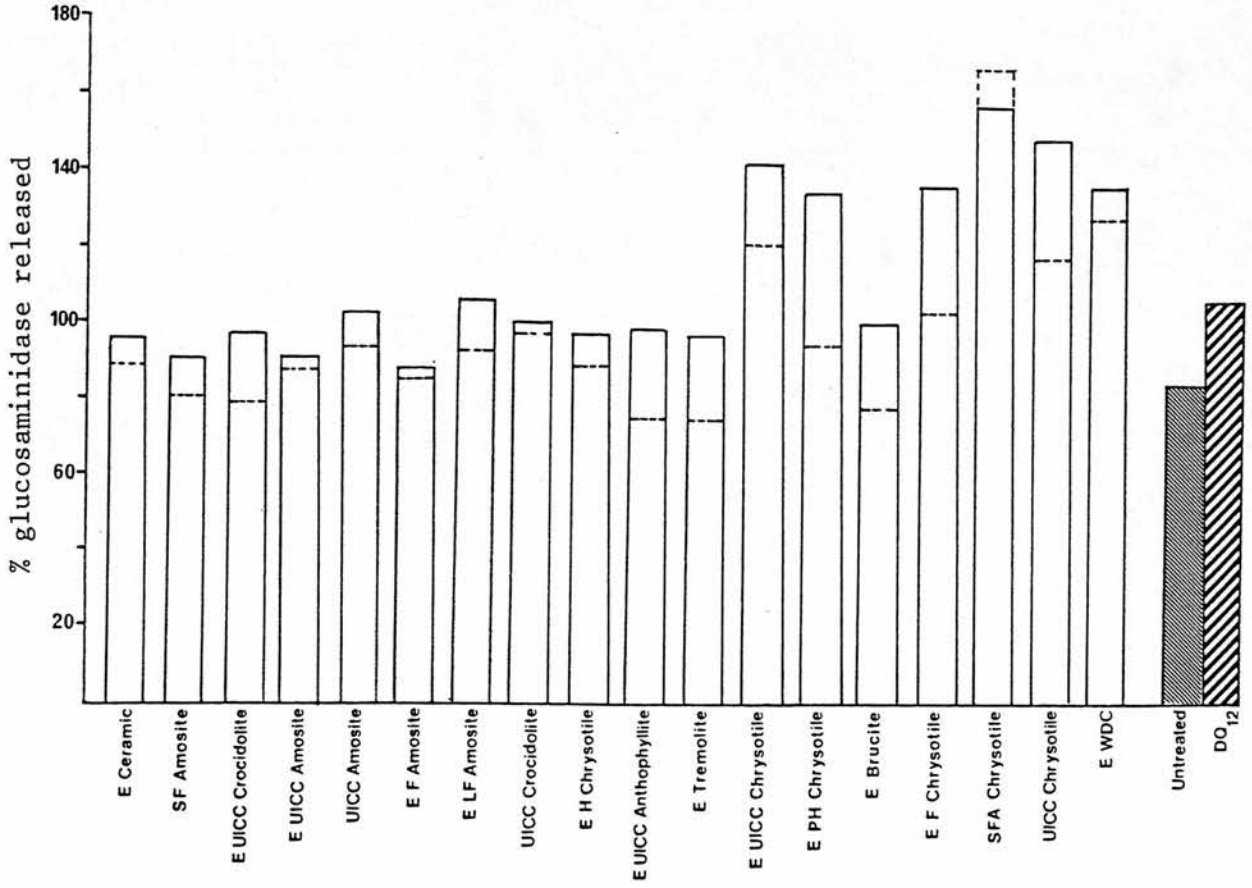

Figure 4.25 Overall Percentage of Glucosaminidase Released Into Medium Following Treatment of $P388D_1$ Cells With Group 1 Dusts.

E ceramic fibre at either 10 or 50 μg/ml for 24 or 48 hrs resulted in an enzyme release pattern that was similar to that of the untreated control population of cells.

4.3.4 The Cytotoxic Ability of Group 2 Dust Samples (the WDC Samples) Towards $P388D_1$ Cells.

The ability of a number of industrially prepared WDC samples (described in Table 4.2) to modify the viability and also enzyme release pattern of $P388D_1$ cells was examined, and UICC chrysotile and E WDC samples described previously for the Group 1 dusts were included for comparison. The results are shown in Tables 4.15 to 4.24; each value is expressed as a percentage of the TiO_2 ± SD (as described previously), and is a mean of the values obtained from at least 3 separate experiments.

4.3.4.1 Percentage Viability.

The viability of the $P388D_1$ cells at 24 and 48 hrs following treatment with 10 and 50 μg/ml of the various WDC fibrous samples is shown in Table 4.15. The E milled chrysotile sample showed a similar degree of cytotoxicity to that observed for the UICC chrysotile sample, at both concentrations of dust at both time points. All of the WDC samples, with the exception of E unextracted WDC, proved more cytotoxic than UICC chrysotile ($p<0.02$ between UICC chrysotile and E WDC at 50 μg/ml for 24 hrs and 10 μg/ml for 48 hrs). E unextracted WDC proved less cytotoxic than the UICC chrysotile sample ($p<0.002$ between UICC chrysotile and E unextracted WDC at 50 μg/ml for 24 hrs and 10 and 50 μg/ml for 48 hrs).

4.3.4.2 Lactate Production.

The production of lactate by the $P388D_1$ cells at 24 and 48 hrs following treatment with 10 and 50 μg/ml of Group 2 dusts is shown in Table 4.16. In general, these samples stimulated a production of lactate which was greater than the level observed for the TiO_2 control at 24 hrs; at the 48 hrs time point the levels were similar to or less than those observed for the TiO_2 control.

Table 4.15 Viability of P388D$_1$ Cells Following Treatment With Group 2 Dusts.

Dust	24 Hrs		48 Hrs	
	10 µg/ml	50 µg/ml	10 µg/ml	50 µg/ml
E Milled Chrysotile	83.6 ± 14.5	34.1 ± 13.9	48.8 ± 11.7	18.4 ± 6.7
E F WDC	49.8 ± 7.8	19.8 ± 7.7	25.4 ± 3.1	10.2 ± 2.5
E Milled WDC	44.1 ± 5.9	24.9 ± 4.1	21.7 ± 4.3	8.2 ± 2.1
E WDC	68.1 ± 10.6	34.3 ± 2.6	29.8 ± 11.1	17.4 ± 5.3
E Heat-Cleaned WDC	54.2 ± 14.6	21.6 ± 5.3	24.4 ± 3.8	9.2 ± 5.5
E Unextracted WDC	94.5 ± 10.6	80.9 ± 13.0	79.0 ± 7.1	60.7 ± 6.1
UICC Chrysotile	82.0 ± 7.9	45.4 ± 8.9	51.0 ± 5.8	20.6 ± 5.0
Untreated Control	105.2 ± 10.6		113.1 ± 14.0	
DQ_{12}	45.2 ± 9.9		28.7 ± 6.9	

Table 4.16 Lactate Production by P388D$_1$ Cells Following Treatment With Group 2 Dusts.

Dust	24 Hrs		48 Hrs	
	10 µg/ml	50 µg/ml	10 µg/ml	50 µg/ml
E Milled Chrysotile	147.4 ± 14.8	130.6 ± 53.7	103.4 ± 4.8	78.2 ± 22.8
E F WDC	130.7 ± 41.6	138.9 ± 59.2	80.2 ± 19.3	70.2 ± 21.0
E Milled WDC	155.4 ± 38.8	124.4 ± 31.4	103.4 ± 9.1	89.9 ± 30.5
E WDC	136.3 ± 12.1	138.7 ± 17.4	129.8 ± 6.8	116.3 ± 31.5
E Heat-Cleaned WDC	156.3 ± 113.3	98.9 ± 42.1	92.9 ± 38.3	52.7 ± 7.5
E Unextracted WDC	112.8 ± 24.6	261.7 ± 192.5	98.4 ± 20.0	157.6 ± 53.6
UICC Chrysotile	123.3 ± 25.0	129.3 ± 37.8	100.8 ± 16.5	78.7 ± 11.9
Untreated Control	100.8 ± 18.5		100.7 ± 23.7	
DQ_{12}	110.9 ± 23.7		84.8 ± 23.7	

Results are expressed as a percentage of the TiO_2 value.

Each figure is a mean of 3 experiments ± SD.

4.3.4.3 LDH Levels.

The measured levels of LDH following treatment of $P388D_1$ cells with Group 2 dust samples are shown in Table 4.17 to 4.20. The loss of intracellular LDH (Table 4.17) was shown to increase with increasing concentration of dust and time of exposure. The treatment of the cells with UICC chrysotile and E milled chrysotile resulted in a loss of intracellular LDH which was similar for both dusts at both concentrations and time points. The remaining WDC samples, with the exception of E unextracted WDC, proved more cytotoxic (according to their ability to reduce the intracellular LDH levels) than the UICC chrysotile sample ($p<0.05$ between E WDC and UICC chrysotile at 10 and 50 μg/ml at 24 and 48 hrs). Treatment of the $P388D_1$ cells with UICC chrysotile resulted in a greater loss of intracellular LDH than treatment with E unextracted WDC ($p<0.05$ between UICC chrysotile and E unextracted WDC at 50 μg/ml for 24 hrs and 10 and 50 μg/ml for 48 hrs). With regard to the total levels of LDH measured per plate following treatment of $P388D_1$ cells with Group 2 dusts (Table 4.18), it was found that the levels tended to remain at approximately 100% of the TiO_2 control value. The levels of LDH in the medium and the overall percentage of enzyme released into the medium following treatment with Group 2 dusts are shown in Table 4.19 and 4.20 respectively. As described previously (Section 4.3.2.4 and 4.3.3.3), comparisons of these values could not be made because of the large standard deviations associated with them.

4.3.4.4 Glucosaminidase Levels.

The effect of treating $P388D_1$ cells with Group 2 dusts on the levels of glucosaminidase observed in the culture system are shown in Tables 4.21 to 4.24 . It can be seen that all of the samples tested, with the exception of E unextracted WDC, had the capacity to reduce the intracellular levels of glucosaminidase (Table 4.21) to below the level observed for the TiO_2 control at 10 and 50 μg/ml at both 24 and 48 hrs time points. The total level of glucosaminidase per plate of $P388D_1$ cells (Table 4.22) following treatment with the Group 2 dusts, with the exception of E unextracted WDC, proved to be

Table 4.17 Intracellular LDH Levels Following Treatment With Group 2 Dusts.

Dust	24 Hrs		48 Hrs	
	10 µg/ml	50 µg/ml	10 µg/ml	50 µg/ml
E Milled Chrysotile	95.1 ± 8.2	53.6 ± 10.5	71.3 ± 10.4	35.3 ± 5.4
E F WDC	74.2 ± 10.4	39.3 ± 11.7	44.7 ± 5.6	25.9 ± 12.0
E Milled WDC	73.8 ± 12.7	43.4 ± 9.2	40.7 ± 9.8	22.4 ± 1.5
E WDC	81.6 ± 6.5	54.4 ± 4.1	52.6 ± 15.2	31.5 ± 3.1
E Heat-Cleaned WDC	72.2 ± 9.5	40.9 ± 2.5	52.1 ± 14.1	23.4 ± 7.1
E Unextracted WDC	93.3 ± 3.0	89.2 ± 5.5	94.2 ± 11.9	81.2 ± 2.1
UICC Chrysotile	91.9 ± 5.4	62.1 ± 7.6	74.0 ± 11.0	39.6 ± 5.8
Untreated Control	111.4 ± 20.5		112.1 ± 7.3	
DQ_{12}	57.9 ± 9.8		41.7 ± 10.4	

Table 4.18 Total LDH Levels Per Plate Following Treatment With Group 2 Dusts.

Dust	24 Hrs		48 Hrs	
	10 µg/ml	50 µg/ml	10 µg/ml	50 µg/ml
E Milled Chrysotile	141.2 ± 17.5	111.2 ± 5.3	110.2 ± 17.5	104.2 ± 22.3
E F WDC	118.0 ± 10.8	118.3 ± 5.9	101.9 ± 17.1	104.1 ± 16.2
E Milled WDC	112.2 ± 9.3	112.2 ± 14.4	105.3 ± 4.4	104.1 ± 12.8
E WDC	107.9 ± 9.3	111.0 ± 4.2	102.7 ± 8.2	99.7 ± 10.8
E Heat-Cleaned WDC	96.0 ± 18.8	99.4 ± 11.0	98.5 ± 7.5	93.6 ± 13.1
E Unextracted WDC	103.9 ± 6.9	102.1 ± 10.7	105.3 ± 11.4	104.9 ± 14.2
UICC Chrysotile	112.5 ± 17.7	108.2 ± 10.7	100.3 ± 15.1	98.3 ± 12.2
Untreated Control	115.9 ± 28.4		104.1 ± 15.9	
DQ_{12}	105.2 ± 7.3		90.9 ± 25.5	

Results are expressed as a percentage of the TiO_2 value.

Each figure is a mean of 3 experiments ± SD.

Table 4.19 LDH Level In Medium Following Treatment With Group 2 Dusts.

Dust	24 Hrs		48 Hrs	
	10 μg/ml	50 μg/ml	10 μg/ml	50 μg/ml
E Milled Chrysotile	649 ± 807	1265 ± 870	849 ± 888	1431 ± 1421
E F WDC	983 ± 703	1796 ± 1363	1327 ± 1374	1741 ± 1845
E Milled WDC	428 ± 328	527 ± 513	372 ± 32	441 ± 85
E WDC	313 ± 230	517 ± 258	314 ± 108	386 ± 81
E Heat-Cleaned WDC	529	1093	391 ± 245	453 ± 189
E Unextracted WDC	336	414	518 ± 1	164 ± 1
UICC Chrysotile	447 ± 279	778 ± 707	374 ± 441	746 ± 934
Untreated Control	59 ± 40		100 ± 50	
DQ_{12}	1590 ± 207		575 ± 659	

Table 4.20 Overall Percentage of LDH Released Into Medium Following Treatment With Group 2 Dusts.

Dust	24 Hrs		48 Hrs	
	10 μg/ml	50 μg/ml	10 μg/ml	50 μg/ml
E Milled Chrysotile	711 ± 36	1112 ± 737	977 ± 984	1515 ± 1683
E F WDC	835 ± 564	1488 ± 1101	1364 ± 1511	1750 ± 1959
E Milled WDC	374 ± 261	551 ± 293	355 ± 16	430 ± 51
E WDC	292 ± 185	469 ± 233	303 ± 94	389 ± 61
E Heat-Cleaned WDC	500	1033	399 ± 271	254 ± 76
E Unextracted WDC	317	400	144 ± 2	170 ± 17
UICC Chrysotile	373 ± 229	676 ± 549	653 ± 1095	794 ± 1044
Untreated Control	64 ± 69		86 ± 57	
DQ_{12}	604 ± 582		686 ± 87	

Results are expressed as a percentage of the TiO_2 value.
Each figure is a mean of 3 experiments ± SD.
The results at 24 hrs for the E heat-cleaned and E unextracted WDC have no SDs; the value shown is from one experiment as no LDH was released by the TiO_2 treated control cells in the remaining 2 experiments.

Table 4.21 Intracellular Glucosaminidase Levels Following Treatment With Group 2 Dusts.

Dust	24 Hrs		48 Hrs	
	10 μg/ml	50 μg/ml	10 μg/ml	50 μg/ml
E Milled Chrysotile	60.0 ± 13.5	7.6 ± 13.2	49.2 ± 10.1	15.9 ± 14.1
E F WDC	32.4 ± 9.8	6.3 ± 10.9	24.2 ± 21.4	12.4 ± 10.9
E Milled WDC	33.9 ± 23.6	11.6 ± 14.7	0.0 ± 0.0	0.0 ± 0.0
E WDC	55.8 ± 28.3	16.3 ± 22.7	14.9 ± 25.9	0.0 ± 0.0
E Heat-Cleaned WDC	66.0 ± 3.4	31.6 ± 4.9	48.3 ± 6.8	28.5 ± 1.1
E Unextracted WDC	148.7 ± 20.2	112.3 ± 21.1	121.0 ± 93.5	119.8 ± 31.0
UICC Chrysotile	78.2 ± 11.2	21.5 ± 19.1	62.3 ± 17.4	16.1 ± 15.5
Untreated Control	207.1 ± 32.1		291.9 ± 110.8	
DQ_{12}	77.6 ± 18.1		54.8 ± 23.5	

Table 4.22 Total Glucosaminidase Levels Per Plate Following Treatment With Group 2 Dusts.

Dust	24 Hrs		48 Hrs	
	10 μg/ml	50 μg/ml	10 μg/ml	50 μg/ml
E Milled Chrysotile	96.2 ± 12.9	67.6 ± 15.3	85.5 ± 13.0	69.0 ± 10.8
E F WDC	90.4 ± 13.2	82.4 ± 10.5	79.0 ± 15.6	67.1 ± 15.8
E Milled WDC	87.6 ± 6.4	87.9 ± 11.2	68.2 ± 22.4	74.0 ± 19.6
E WDC	106.9 ± 6.1	94.7 ± 6.4	99.5 ± 13.3	81.4 ± 5.4
E Heat-Cleaned WDC	95.0 ± 5.4	79.6 ± 3.9	80.2 ± 5.9	69.2 ± 11.6
E Unextracted WDC	137.8 ± 11.2	98.5 ± 21.9	110.2 ± 32.8	109.3 ± 11.3
UICC Chrysotile	102.0 ± 8.3	66.4 ± 9.2	88.4 ± 7.8	64.6 ± 7.7
Untreated Control	170.8 ± 25.2		162.5 ± 23.0	
DQ_{12}	98.8 ± 10.7		100.9 ± 35.8	

Results are expressed as a percentage of the TiO_2 value.

Each figure is a mean of 3 experiments ± SD.

similar or less than the value obtained for the TiO_2 control. With regard to the quantity of glucosaminidase present in the culture medium (Table 4.23), the Group 2 dusts, with the exception of E unextracted WDC, stimulated a level which was greater than the level induced by TiO_2 at 24 hrs; but the level became equivalent to the value for the TiO_2 treated cells at 48 hrs. A similar trend was observed for the overall percentage of glucosaminidase per plate released into the medium (Table 4.24). The Group 2 dusts, with the exception of E unextracted WDC, induced a greater release of enzyme than the TiO_2 control at 24 hrs, but was still higher than the percentage released by the TiO_2 treated $P388D_1$ cells.

4.3.5 The Inter-Relationship Between Cell Viability and Enzyme Levels During the Interaction of $P388D_1$ Cells with Group 1 and Group 2 Dust Samples.

In order to establish the relationship between cell viability and also the various enzyme levels during the interaction of the $P388D_1$ cells with the different dust samples, the data for each parameter measured in the cytotoxicty experiments for Group 1 and Group 2 dusts (Tables 4.6 to 4.14 and Tables 4.15 to 4.24) were combined. The various parameters were examined in pairs, and correlation coefficients for any linear associations were established. The correlation coefficients obtained for each paired comparison for dust concentrations of 10 and 50 μg/ml following 24 and 48 hrs of exposure of $P388D_1$ cells are shown in Figures 4.26 to 4.29. A correlation coefficient of <-0.396 or >0.396 denotes a statistical signficance of $p<0.05$.

Significantly positive correlation coefficients were obtained for the associations between the measurements for cell viability and intracellular LDH, cell viability and intracellular glucosaminidase and also cell viability and the total glucosaminidase levels per plate for $P388D_1$ cells at 24 and 48 hrs following treatment with 10 or 50 μg/ml of fibres ($p<0.001$); and a negative correlation coefficient was obtained for the association between cell viability and the overall percentage of glucosaminidase released following the 4 treatment conditions ($p<0.001$). The intracellular levels of the enzymes LDH and glucosaminidase also showed a significantly positive association

Table 4.23 Glucosaminidase Levels in Medium Following Treatment With Group 2 Dusts.

Dust	24 Hrs		48 Hrs	
	10 μg/ml	50 μg/ml	10 μg/ml	50 μg/ml
E Milled Chrysotile	144.9 ± 50.4	147.1 ± 74.8	102.7 ± 23.2	93.6 ± 17.5
E F WDC	167.4 ± 73.8	183.1 ± 89.6	104.4 ± 32.1	92.8 ± 26.7
E Milled WDC	169.5 ± 35.5	181.6 ± 41.3	111.3 ± 22.3	99.9 ± 22.8
E WDC	172.3 ± 40.1	190.6 ± 39.9	118.8 ± 20.9	101.4 ± 21.1
E Heat-Cleaned WDC	135.7 ± 20.5	184.0 ± 34.6	106.2 ± 18.3	97.6 ± 18.5
E Unextracted WDC	117.8 ± 8.0	130.2 ± 17.5	105.3 ± 16.6	103.6 ± 22.2
UICC Chrysotile	137.1 ± 22.2	131.1 ± 26.8	102.7 ± 13.5	92.3 ± 21.8
Untreated Control	128.0 ± 31.9		112.8 ± 26.4	
DQ_{12}	112.1 ± 22.0		83.1 ± 12.9	

Table 4.24 Overall Percentage of Glucosaminidase Released Into Medium Following Treatment With Group 2 Dusts.

Dust	24 Hrs		48 Hrs	
	10 μg/ml	50 μg/ml	10 μg/ml	50 μg/ml
E Milled Chrysotile	148.7 ± 33.2	215.8 ± 80.5	124.0 ± 7.6	141.7 ± 14.4
E F WDC	181.5 ± 54.5	216.9 ± 79.1	131.0 ± 14.6	137.8 ± 12.2
E Milled WDC	180.6 ± 32.6	206.6 ± 21.9	136.7 ± 21.7	136.7 ± 21.7
E WDC	162.9 ± 34.9	201.9 ± 29.3	129.0 ± 29.0	136.7 ± 21.7
E Heat-Cleaned WDC	152.8 ± 13.9	222.9 ± 42.0	155.3 ± 18.4	150.7 ± 20.8
E Unextracted WDC	85.7 ± 9.5	112.7 ± 22.1	105.6 ± 54.8	94.6 ± 18.3
UICC Chrysotile	134.8 ± 17.4	201.9 ± 48.4	115.2 ± 8.2	138.5 ± 15.4
Untreated Control	76.7 ± 10.0		66.6 ± 5.5	
DQ_{12}	113.6 ± 36.9		111.5 ± 12.8	

Results are expressed as a percentage of the TiO_2 control.
Each figure is a mean of 3 experiments ± SD.

	viability	1.00				
	LDH in cells	+.90	1.00			
Glucosaminidase	in cells	+.80	+.73	1.00		
	in medium	-.26	-.70	-.33	1.00	
	total	+.70	+.67	+.91	0.00	1.00
	% released	-.76	-.65	-.91	-.63	-.73	1.00
		viability	LDH in cells	in cells	in medium	total	% released
				Glucosaminidase			

Figure 4.26 Correlation Coefficients for Relationship Between Viability and Enzyme Assays Following Treatment With Group 1 and 2 (10 µg/ml) Dusts for 24 Hrs.

	viability	1.00				
	LDH in cells	+.97	1.00			
Glucosaminidase	in cells	+.88	+.88	1.00		
	in medium	-.42	-.40	-.42	1.00	
	total	+.77	+.77	+.85	0.00	1.00
	% released	-.84	-.82	-.85	+.67	-.63	1.00
		viability	LDH in cells	in cells	in medium	total	% released
				Glucosaminidase			

Figure 4.27 Correlation Coefficients for Relationships Between Viability and Enzyme Assays Following Treatment With Group 1 and 2 (50 µg/ml) Dusts for 24 Hrs.

		viability	LDH in cells	Glucosaminidase: in cells	Glucosaminidase: in medium	Glucosaminidase: total	Glucosaminidase: % released
viability		1.00					
LDH in cells		+.92	1.00				
Glucosaminidase	in cells	+.83	+.81	1.00			
	in medium	+.43	+.43	+.29	1.00		
	total	+.84	+.80	+.89	+.59	1.00	
	% released	-.83	-.73	-.81	-.17	-.76	1.00

Figure 4.28 Correlation Coefficients for Relationship Between Viability and Enzyme Assays Following Treatment With Group 1 and 2 (10 µg/ml) Dusts for 48 Hrs.

		viability	LDH in cells	Glucosaminidase: in cells	Glucosaminidase: in medium	Glucosaminidase: total	Glucosaminidase: % released
viability		1.00					
LDH in cells		+.95	1.00				
Glucosaminidase	in cells	+.89	+.87	1.00			
	in medium	+.55	+.44	+.49	1.00		
	total	+.85	+.78	+.88	+.76	1.00	
	% released	-.86	-.83	-.86	-.32	-.77	1.00

Figure 4.29 Correlation Coefficients for Relationships Between Viability and Enzyme Assays Following Treatment With Group 1 and 2 Dusts (50 µg/ml) for 48 Hrs.

($p<0.001$) following treatment of the cells with 10 or 50 μg/ml of dust at both time points. The intracellular glucosaminidase levels also showed a significantly positive association with the total levels of glucosaminidase per plate ($p<0.001$) and a significant negative association with the overall percentage of glucosaminidase released ($p<0.001$) following treatment of cells with 10 or 50 μg/ml of fibres for 24 or 48 hrs. These results have therefore shown that "the greater the cytotoxic capacity of a dust, the greater is its ability to reduce the cell viability, the intracellular LDH and glucosaminidase levels, and also increase the percentage of glucosamindase released into the medium".

4.3.6 The Effect of Fibre Length on P388D$_1$ Cell Viability and Enzyme Release.

One of the aims of this study was to establish the relationship between the fibre length content of an asbestos sample and its ability to modify the viability and enzyme release pattern of P388D$_1$ cells. 13 fibrous dust samples were selected because of their purity for use in this part of the study and they are described in Section 4.2.4. The data concerning the ability of these samples to alter the P388D$_1$ cell viability and enzyme levels after exposure for 24 and 48 hrs to 10 or 50 μg/ml of each sample was obtained from Section 4.3.3. This cytotoxicity data was compared with the number of fibres of greater than various length thresholds (from 1 to 20 μm in 1 μm steps) that were present per unit weight in each sample, and correlation coefficients for the associations between each paired comparison were calculated as described in Section 4.2.4. Before describing this data, however, the relationship between the viability and enzyme release patterns for these dusts is presented, in order to establish whether the associations were similar to those described in Section 4.3.5.

4.3.6.1 Inter-Relationship Between Viability and Enzyme Release From P388D$_1$ Cells Treated With the 13 Selected Fibrous Samples.

In order to establish the association between viability and enzyme release following the treatment of P388D$_1$ cells with the sub-set

of 13 fibrous dusts at concentrations of 10 or 50 μg/ml for 24 and 48 hrs, the various parameters were compared in pairs as described in Section 4.3.5. The correlation coefficients obtained as a result of these comparisons are shown in Figures 4.30 to 4.33; a correlation coefficient of <-0.553 or >0.553 denotes a statistical significance of $p<0.05$.

In general, at the 48 hrs time point following treatment with 10 and 50 μg/ml of dust and at 24 hrs following treatment with 50 μg/ml (Figures 4.31 to 4.33), the relationship between cellular viability and LDH and glucosaminidase levels proved to be similar to the relationship described in Section 4.3.5, in that the value for cellular viability showed a positive association with the levels obtained for intracellular LDH and glucosaminidase and total glucosaminidase levels ($p<0.05$) and a negative association with the percentage of glucosaminidase released into the medium ($p<0.05$). At the 24 hrs time point, following treatment of $P388D_1$ cells with a 10 μg/ml concentration of dust (Figure 4.30), the trend was different, in that the correlation coefficients for the relationships between cellular viability and intracellular LDH and glucosaminidase, and glucosaminidase release were not significant. The relationship between cellular viability and the intracellular LDH levels (10 μg/ml of dust for 24 hrs) is shown in Figure 4.34; it can be observed that the intracellular LDH levels do not change at the same rate as the values for the viability, following dust treatment, and thus no significant association between these two variables could be seen. The relationship between percentage viability and intracellular glucosaminidase is shown in Figure 4.35, and between percentage viability and glucosaminidase release in Figure 4.36. In neither instance was it possible to find a statistically significant linear or curved relationship between these variables.

4.3.6.2 The Relationship Between Viability, Enzyme Release and Fibre Length.

The correlation coefficients obtained as a result of the paired comparisons between viability, enzyme assays and fibre length are shown in Tables 4.25 to 4.28. A correlation coefficient of <-0.553 or >0.553 denotes a statistical significance of $p<0.05$. An asterisk

		viability	LDH in cells	Glucosaminidase: in cells	Glucosaminidase: in medium	Glucosaminidase: total	Glucosaminidase: % released
viability		1.00					
LDH in cells		+.45	1.00				
Glucosaminidase	in cells	+.52	+.42	1.00			
	in medium	+.32	+.77	+.30	1.00		
	total	+.61	+.61	+.96	+.50	1.00	
	% released	-.36	-.10	-.79	+.28	-.66	1.00

Figure 4.30 Correlation Coefficients for Relationships Between Viability and Enzyme Assays Following Treatment With "13" Fibrous Samples (10 μg/ml) for 24 Hrs.

		viability	LDH in cells	Glucosaminidase: in cells	Glucosaminidase: in medium	Glucosaminidase: total	Glucosaminidase: % released
viability		1.00					
LDH in cells		+.91	1.00				
Glucosaminidase	in cells	+.80	.79	1.00			
	in medium	+.27	+.44	+.51	1.00		
	total	+.82	+.88	+.91	+.60	1.00	
	% released	-.60	-.48	-.48	+.19	-.46	1.00

Figure 4.31 Correlation Coefficients for Relationships Between Viability and Enzyme Assays Following Treatment With "13" Fibrous Samples (50 μg/ml) for 24 Hrs.

		viability	LDH in cells	Glucosaminidase: in cells	Glucosaminidase: in medium	Glucosaminidase: total	Glucosaminidase: % released
viability		1.00					
LDH in cells		+.82	1.00				
Glucosaminidase	in cells	+.60	+.62	1.00			
	in medium	+.52	+.50	+.17	1.00		
	total	+.78	+.80	+.79	+.70	1.00	
	% released	-.73	-.60	-.70	-.17	-.64	1.00

Figure 4.32 Correlation Coefficients for Relationships Between Viability and Enzyme Release Following Treatment With "13" Fibrous Samples (10 μg/ml) for 48 Hrs.

		viability	LDH in cells	Glucosaminidase: in cells	Glucosaminidase: in medium	Glucosaminidase: total	Glucosaminidase: % released
viability		1.00					
LDH in cells		+.92	1.00				
Glucosaminidase	in cells	+.93	+.86	1.00			
	in medium	+.78	+.76	+.76	1.00		
	total	+.88	+.84	+.93	+.93	1.00	
	% released	-.73	-.62	-.84	-.35	-.61	1.00

Figure 4.33 Correlation Coefficients for Relationships Between Viability and Enzyme Assays Following Treatment With "13" Fibrous Samples (50 μg/ml) for 48 Hrs.

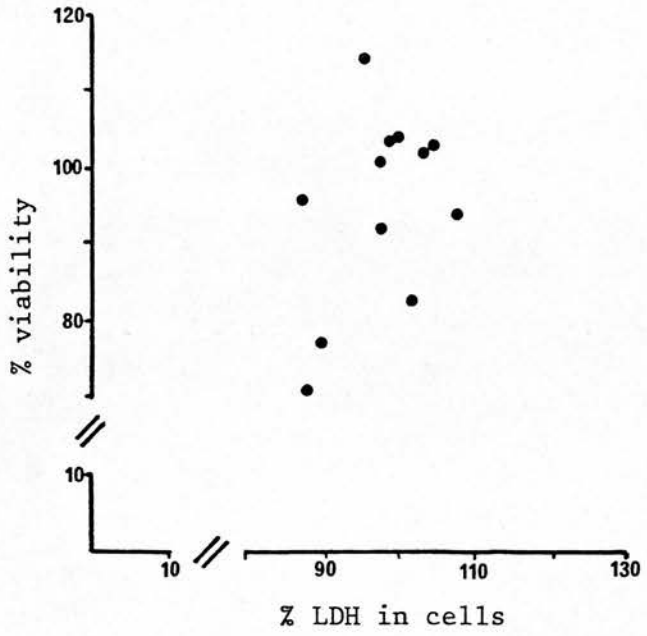

Figure 4.34 Relationship Between Viability and Intracellular LDH 24 Hrs Following Treatment With "13" Fibrous Samples (10 μg/ml).

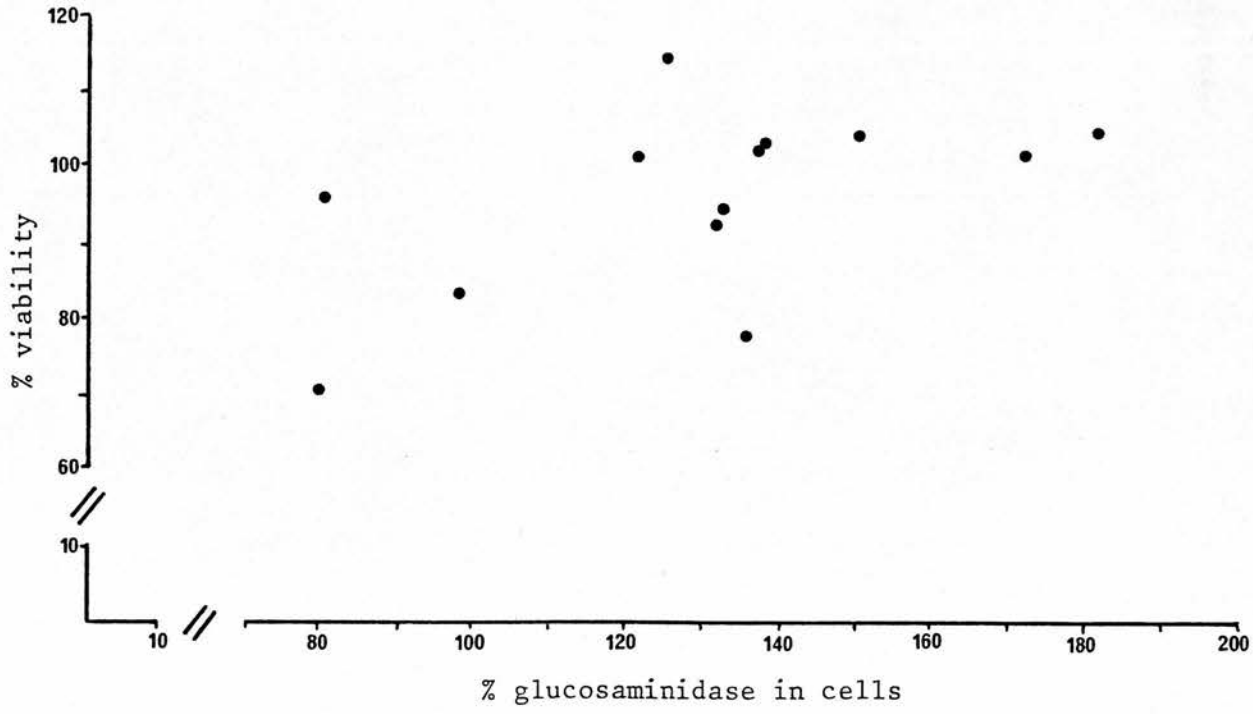

Figure 4.35 Relationship Between Viability and Intracellular Glucosaminidase 24 Hrs Following Treatment With "13" Fibrous Samples (10 μg/ml).

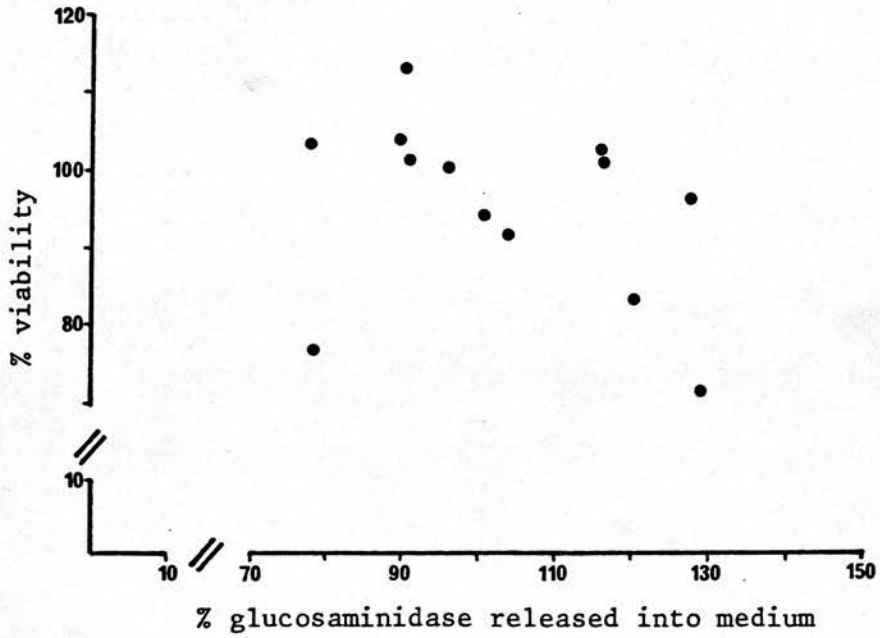

Figure 4.36 Relationship Between Viability and Glucosaminidase Release 24 Hrs Following Treatment With "13" Fibrous Samples (10 μg/ml).

denotes the fibre length threshold for which the maximum correlation coefficient value for each cytotoxicity assay parameter occurs.

a) Percentage Viability.

With regard to the relationship between percentage viability and the total fibre number per unit weight, the association was not significant at the 10 or 50 μg/ml treatment at the 24 or 48 hrs time points. This observation is illustrated in Figure 4.37 which comprises a comparison of the number of fibres/10^{-10} g and percentage viability following treatment of $P388D_1$ cells at a dust concentration of 50 μg/ml for 48 hrs. However, it was apparent that the degree of significance for the association between percentage viability and fibre length increased as the value for the fibre length threshold increased. At the 24 hrs time point the maximum degree of correlation for these two parameters occurred at the 7 or 8 μm length threshold for the 10 μg/ml concentration ($p<0.02$), and 8 μm for the 50 μg/ml concentration ($p<0.01$). At 48 hrs a maximum correlation occurred between viability and the 8 μm length threshold for the 10 and 50 μg/ml concentrations of dust ($p<0.01$). The association between viability and fibre length was negative in value, thus a decrease in cellular viability was observed as the presence of the number of fibres of greater than 8 μm in length on the culture plate increased. The relationship between the number of fibres of greater than 8 μm in length and the percentage viability is shown in Figures 4.38 to 4.41. From the plots for the four graphs a linear relationship can be observed between the variables. Although included in the results, one point on each graph (representing E brucite) has been circled as the result for this fibrous sample did not coincide with the observed relationship between the remaining dust samples and percentage viability. It must be noted that in Figure 4.41 (50 μg/ml for 48 hrs) a curved relationship between these variables may be seen. In order to investigate this situation further, correlation coefficients were calculated for the non-linear associations between fibre length and viability for the "50 μg/ml for 48 hrs" set of data (method described in Section 4.2.4). The correlation coefficients for these non-linear associations (Table 4.29) displayed a similar trend to the one observed for the assumed linear associations; the value for the correlation coefficients increased as the fibre length

Table 4.25 Correlation Coefficients for Relationship Between the No of Fibres Greater Than Each Length Category and Their Biological Activity 24 Hrs Following Treatment of $P388D_1$ Cells With Dust (10 μg/ml).

		Fibre Length Category (μm)												
		0	1	2	3	4	5	6	7	8	9	10	15	20
viability		-.41	-.52	-.64	-.66	-.64	-.65	-.63	-.68*	-.68	-.67	-.64	-.16	-.30
LDH in cells		+.03	-.03	-.18	-.19	-.10	-.18	-.23	-.33	-.40	-.46	-.58	-.75	-.79*
Glucosaminidase	in cells	-.63	-.70*	-.70*	-.66	-.54	-.53	-.58	-.60	-.64	-.49	-.48	-.26	-.48
	total	-.51	-.59	-.63	-.60	-.49	-.50	-.54	-.61	-.65*	-.55	-.55	-.35	-.55
	% released	+.63	+.75	+.78*	+.78*	+.69	+.68	+.75	+.71	+.75	+.58	+.51	+.25	+.40

*Denotes the fibre length threshold which shows the greatest association with each cytotoxicity parameter.

Table 4.26 Correlation Coefficients for Relationship Between the No of Fibres Greater Than Each Fibre Length Category and Their Biological Activity 24 Hrs Following Treatment of $P388D_1$ Cells With Dust (50 μg/ml).

		Fibre Length Category (μm)												
		0	1	2	3	4	5	6	7	8	9	10	15	20
viability		-.30	-.43	-.60	-.62	-.60	-.62	-.63	-.68	-.71*	-.67	-.64	-.21	-.16
LDH in cells		-.09	-.21	-.38	-.43	-.36	-.43	-.47	-.54	-.59	-.58	-.62*	-.36	-.32
Glucosaminidase	in cells	-.30	-.39	-.47	-.45	-.37	-.36	-.42	-.47	-.55*	-.45	-.45	-.30	-.24
	total	-.31	-.39	-.48	-.48	-.38	-.41	-.45	-.51	-.56*	-.51	-.52	-.27	-.27
	% released	+.44	+.63	+.81	+.87	+.87	+.91	+.96*	+.94	+.96*	+.91	+.89	+.40	+.44

*Denotes the fibre length threshold which shows the greatest association with each cytotoxicity parameter.

Table 4.27 Correlation Coefficients for Relationship Between the No of Fibres Greater Than Each Fibre Length Category and Their Biological Activity, 48 Hrs Following Treatment of $P388D_1$ Cells With Dust (10 μg/ml).

		Fibre Length Category (μm)												
		0	1	2	3	4	5	6	7	8	9	10	15	20
viability		-.50	-.63	-.78	-.81	-.77	-.79	-.80	-.87	-.88*	-.82	-.76	-.25	-.30
LDH in cells		-.41	-.51	-.62	-.66	-.59	-.65	-.74	-.82*	-.79	-.78	-.73	-.30	-.37
Glucosaminidase	in cells	-.63	-.68*	-.65	-.61	-.48	-.48	-.54	-.56	-.57	-.43	-.40	-.17	-.31
	total	-.48	-.56	-.64	-.64	-.54	-.57	-.62	-.67	-.69*	-.59	-.63	-.27	-.29
	% released	+.54	+.66	+.72*	+.69	+.69	+.63	+.66	+.72*	+.70	+.60	+.45	+.03	+.09

*Denotes the fibre length threshold which shows the greatest association with each cytotoxicity parameter.

Table 4.28 Correlation Coefficients for Relationship Between the No of Fibres Greater Than Each Fibre Length Category and Their Biological Activity, 48 Hrs Following Treatment of $P388D_1$ Cells With Dust (50 μg/ml).

		Fibre Length Category (μm)												
		0	1	2	3	4	5	6	7	8	9	10	15	20
viability		-.23	-.42	-.63	-.66	-.68	-.69	-.72	-.75	-.78*	-.76	-.71	-.27	-.19
LDH in cells		-.10	-.23	-.41	-.47	-.47	-.50	-.58	-.62	-.66*	-.65	-.62	-.34	-.20
Glucosaminidase	in cells	-.31	-.46	-.63	-.64	-.60	-.62	-.68	-.75	-.78*	-.75	-.70	-.34	-.28
	total	-.13	-.25	-.41	-.42	-.39	-.40	-.45	-.51	-.56*	-.54	-.53	-.25	-.14
	% released	+.54	+.68	+.81	+.79	+.76	+.76	+.80	+.88*	+.87	+.79	+.71	+.27	+.29

*Denotes the fibre length threshold which shows the greatest association with each cytotoxicity parameter.

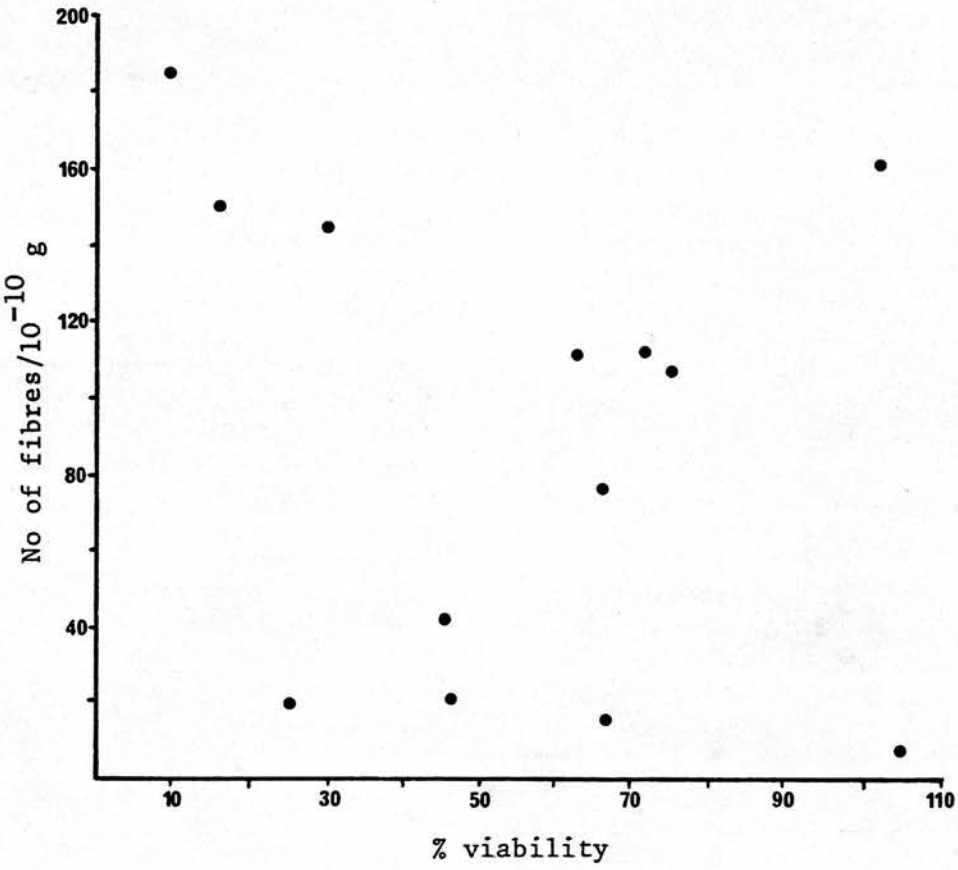

Figure 4.37 Relationship Between Total Fibre Number and Viability 48 Hrs Following Treatment With Dust (50 μg/ml).

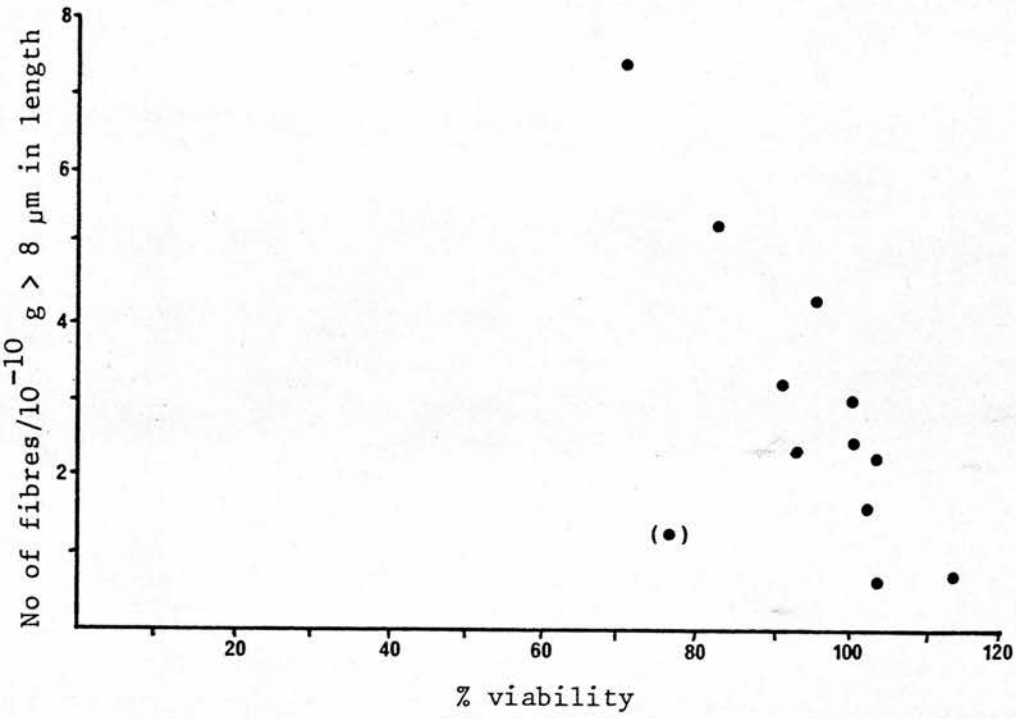

Figure 4.38 Relationship Between Number of Fibres Greater Than 8 μm in Length and Viability, 24 Hrs Following Treatment With Dust (10 μg/ml).

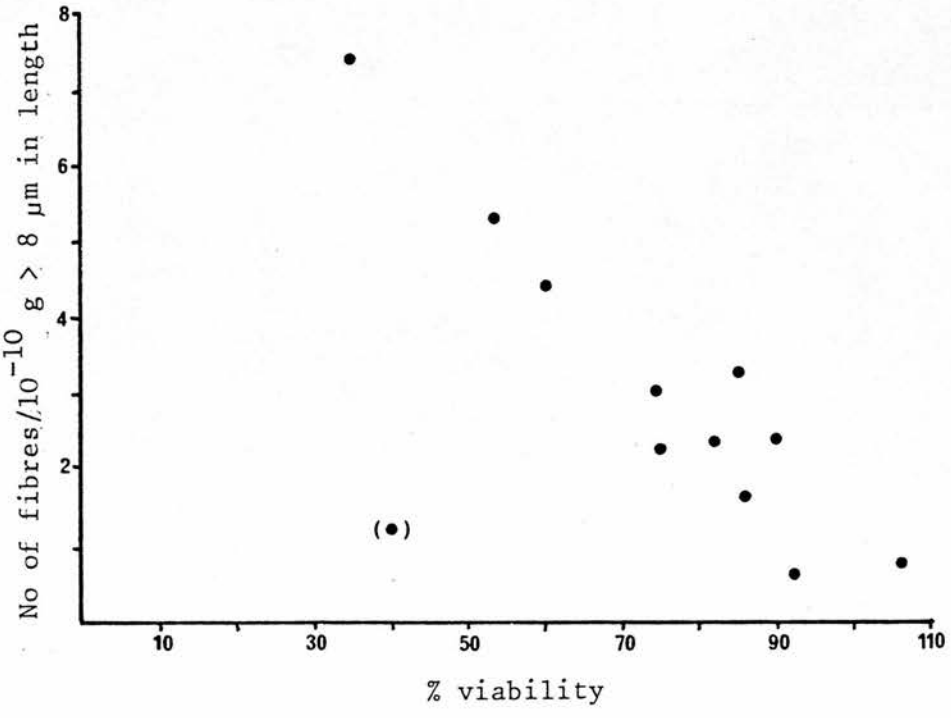

Figure 4.39 Relationship Between Number of Fibres Greater Than 8 μm in Length and Viability, 24 Hrs Following Treatment With Dust (50 μg/ml).

( ) denotes E brucite sample.

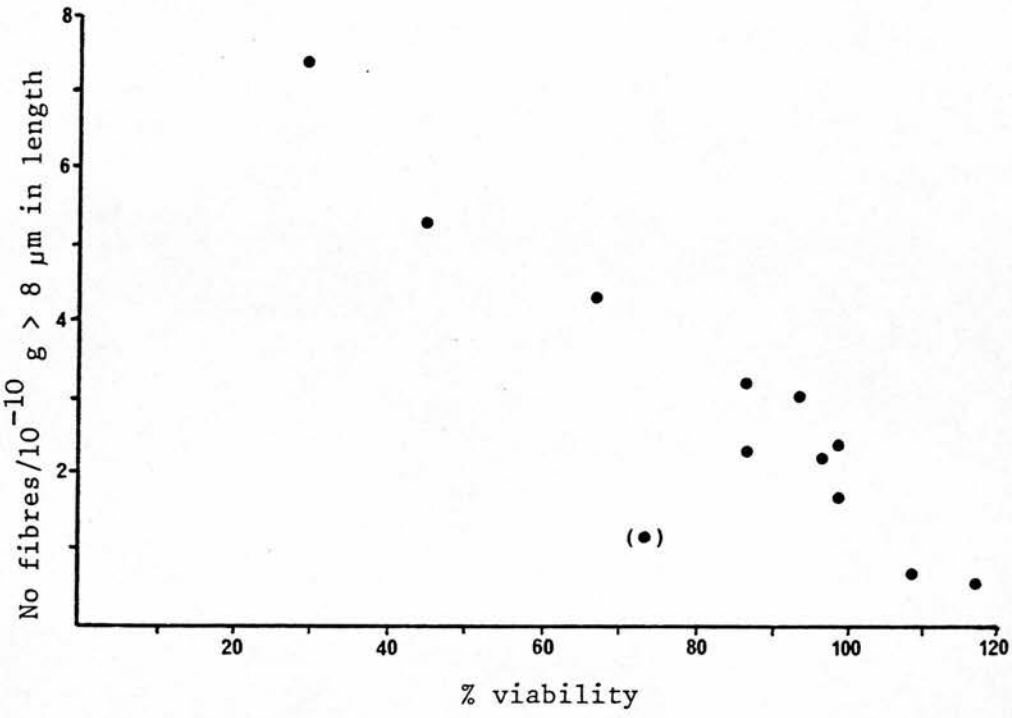

Figure 4.40 Relationship Between Number of Fibres Greater Than 8 μm in Length and Viability, 48 Hrs Following Treatment With Dust (10 μg/ml).

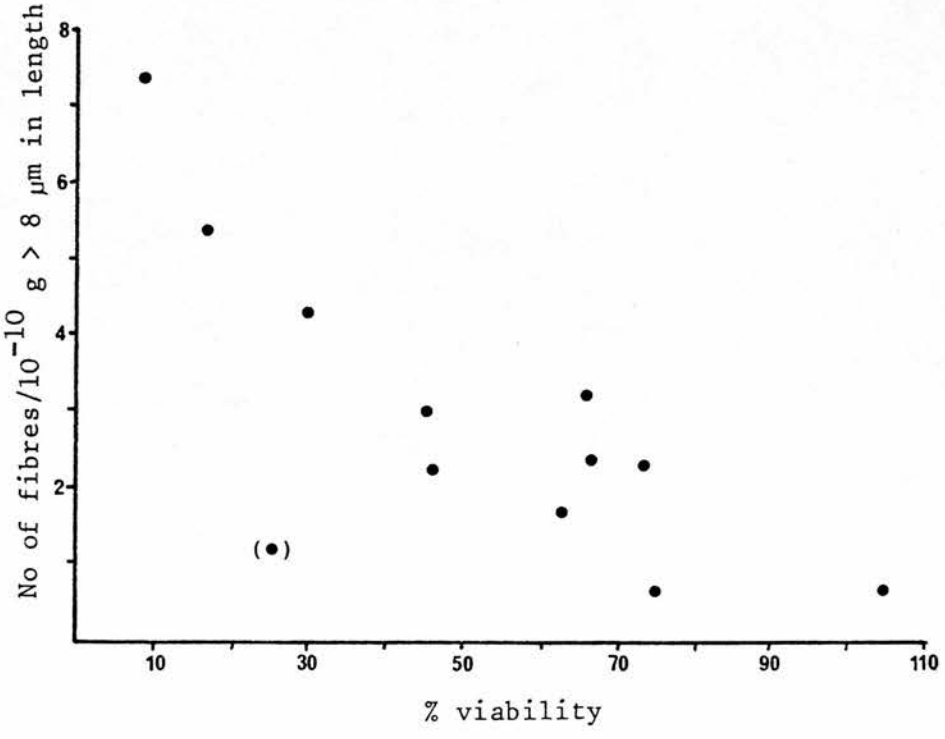

Figure 4.41 Relationship Between Number of Fibres Greater Than 8 μm in Length and Viability, 48 Hrs Following Treatment With Dust (50 μg/ml).

( ) denotes E brucite sample.

Table 4.29 Correlation Coefficients for Comparisons of the Non-Linear Relationships Between "Number of Fibres Greater Than Stated Length Category" and "Viability" 48 Hrs Following Treatment of $P388D_1$ Cells With Dust (50 µg/ml).

Fibre Length Category (µm)	Correlation Coefficient
0	+.26
1	+.35
2	+.62
5	+.68
7	+.74
8	+.72
9	+.75
10	+.73
15	+.12
20	-.10

threshold increased in value. A peak value occurred at the 9 μm length rather than the 8 μm length threshold noted for the assumed linear associations. The value of each correlation coefficient for the non-linear association was always lower than the corresponding value for the assumed linear association, and thus it can be concluded that the linear association provided the "best fit" for the data.

b) Intracellular LDH.

The maximum values obtained for those correlation coefficients relating intracellular LDH and fibre length (Tables 4.25 to 4.28) proved to be more variable at the different concentrations and time points than those obtained for the relationship between viability and fibre length. At the 24 hrs time point for the 10 μg/ml concentration, the best correlation between these variables was obtained for those fibres longer than 20 μm ($p<0.01$); at 50 μg/ml the best correlation was obtained at 10 μm ($p<0.01$). At 48 hrs the length at which the maximum correlation occurred was 7 μm for the 10 μg/ml concentration ($p<0.001$) and 8 μm for the 50 μg/ml concentration ($p<0.01$). The relationships between intracellular LDH and the number of fibres longer than 7 or 8 μm for the 10 and 50 μg/ml concentrations respectively at the 48 hrs time point are shown in Figures 4.42 and 4.43. The relationship depicted in Figure 4.42 (10 μg/ml for 48 hrs) proved linear, however the relationship described in Figure 4.43 (50 μg/ml for 48 hrs) could be termed either linear or non-linear. A further investigation of this relationship demonstrated that the linear association gave the "best line of fit" for the data (data not shown).

c) Glucosaminidase.

With regard to the relationships between the intracellular levels of glucosaminidase and fibre length (Tables 4.25 to 4.28), all of the correlation coefficients showed that the association between these variables was negative. A maximum association occurred between the intracellular glucosaminidase and the number of fibres of greater than 1 or 2 μm in length for the 10 μg/ml concentration at 24 and 48 hrs ($p<0.02$); although a peak value for the association between these variables occurred at the 8 μm fibre length threshold

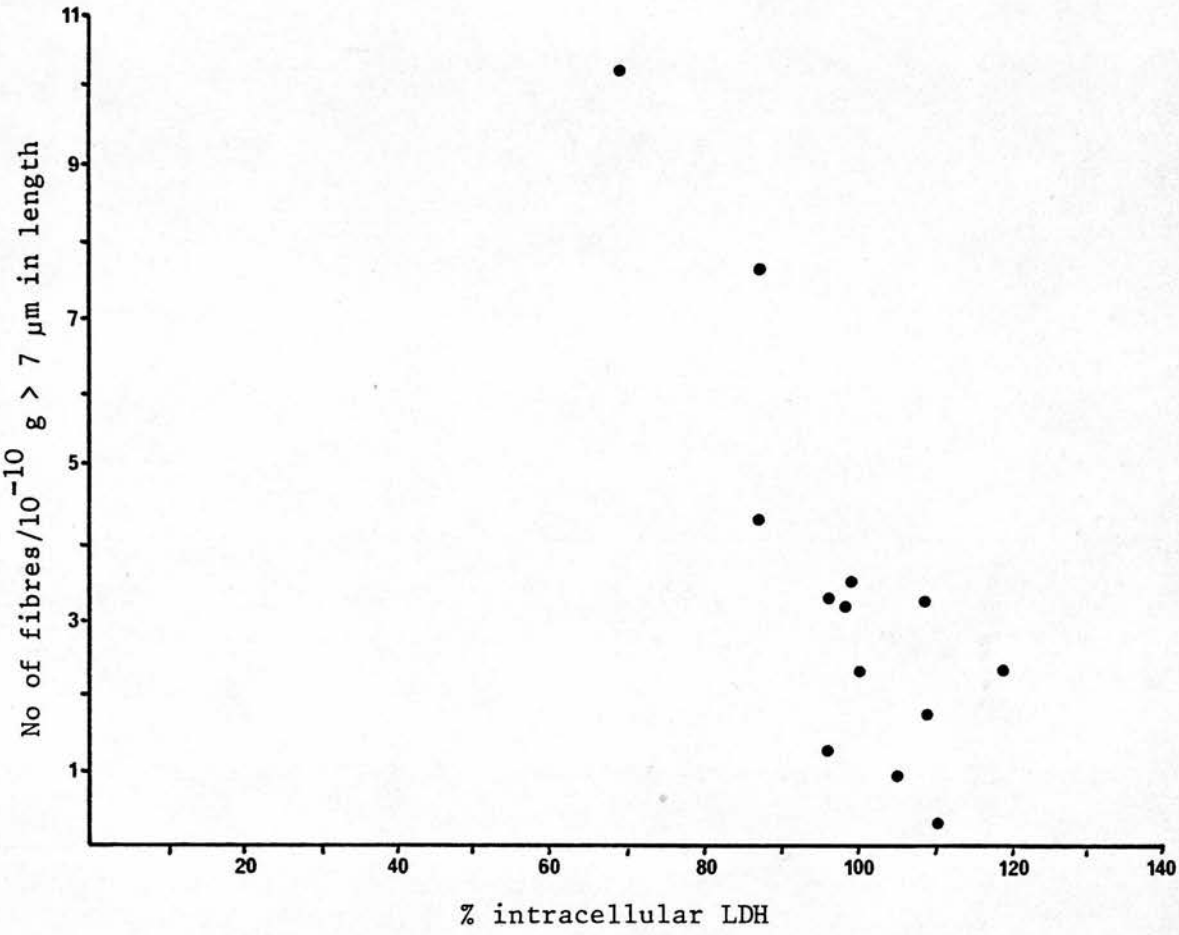

Figure 4.42 Relationship Between Number of Fibres Greater Than 7 μm in Length and Intracellular LDH Levels 48 Hrs Following Treatment With Dust (10 μg/ml).

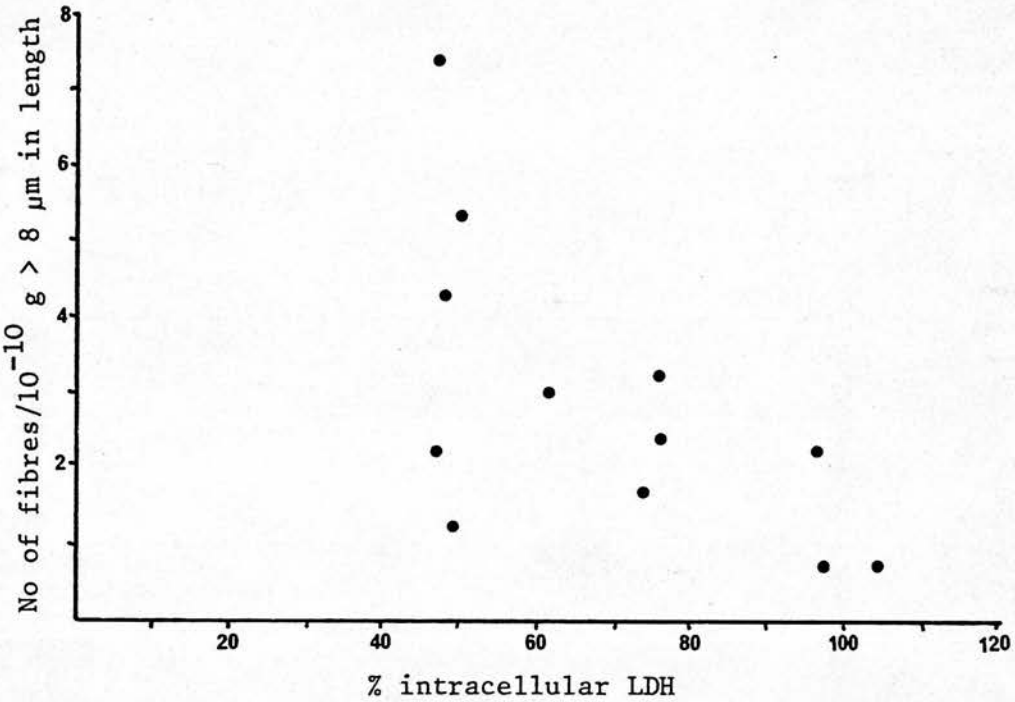

Figure 4.43 Relationship Between Number of Fibres Greater Than 8 μm in Length and Intracellular LDH Levels 48 Hrs Following Treatment With Dust (50 μg/ml).

for the 50 μg/ml concentration at 24 and 48 hrs time points ($p<0.05$ and $p<0.01$ respectively). A similar trend was observed for the association between the overall percentage of glucosaminidase released per plate and fibre length. Maximum correlation coefficients for the positive associations between these variables occurred at the 2 and 3 μm fibre length thresholds for the 10 μg/ml concentration for 24 hrs ($p<0.01$), and 6 to 8 μm for 50 μg/ml for 24 hrs ($p<0.02$), 2 and 7 μm at the 10 μg/ml concentration for 48 hrs and 7 μm only at 50 μg/ml for 48 hrs ($p<0.02$). With regard to the relationship between the total level of glucosaminidase per plate and fibre length, however, negative associations for these variables were consistently observed for the numbers of fibres of greater than 8 μm at both dust concentrations and time points ($p<0.05$). These results would indicate that the relationship between the cytotoxic capacity of a dust sample with respect to its ability to modify glucosaminidase release, and its constituent fibre length content is highly complex.

4.3.7 The Ability of Dust Samples to Increase the Occurrence of $P388D_1$ Cells Containing More Than One Nucleus.

Following the morphological observation that the treatment of $P388D_1$ cells with UICC crocidolite or amosite resulted in an increase in the occurrence of cells containing more than one nucleus (Section 4.3.1), a number of preliminary experiments were carried out in an attempt to quantify the occurrence of these multinucleate cells. The induction of cells possessing more than one nucleus, following treatment with a dose of 20 or 100 μg/plate of various fibrous and non-fibrous dust samples for 24 or 48 hrs is shown in Table 4.30. The number of cells containing more than one nucleus is expressed as a percentage of the total population of cells, and each value is a mean of the results obtained from 3 separate experiments ± SD.

The exposure of the $P388D_1$ cells for 24 hrs to a concentration of either 20 or 100 μg/plate of either particulate or fibrous dust did not cause a significant increase in the number of cells containing more than one nucleus. An apparent reduction in multinucleate cells was observed following TiO_2 treatment, but this may have been due to an inaccurate assessment of the cell population by virtue of the

Table 4.30 The Incidence of Multinucleated $P388D_1$ Cells Following Treatment With Various Dust Samples.

Treatment	% of cells containing more than one nucleus after exposure to dust for various periods of time: 0 Time	24 Hrs	48 Hrs
Control	4.9 ± 2.1	7.4 ± 3.7	3.8 ± 3.7
UICC Crocidolite 20 µg	-	11.8 ± 4.4	15.5 ± 4.7
UICC Crocidolite 100 µg	-	11.3 ± 0.8	26.7 ± 4.9
UICC Amosite 20 µg	-	7.0 ± 1.2	14.4 ± 6.8
UICC Amosite 100 µg	-	8.5 ± 4.0	25.5 ± 8.8
UICC Chrysotile 20 µg	-	13.7 ± 4.0	28.6 ± 13.7
UICC Chrysotile 100 µg	-	11.0 ± 6.8	24.0 ± 14.0
DQ_{12} 20 µg	-	6.1 ± 0.9	2.3 ± 1.4
DQ_{12} 100 µg	-	8.4 ± 3.2	5.7 ± 6.3
TiO_2 20 µg	-	2.9 ± 0.4	4.6 ± 2.4
TiO_2 100 µg	-	2.2 ± 1.9	4.8 ± 4.8
Hetton Coal 20 µg	-	4.1 ± 1.4	4.9 ± 3.5
Hetton Coal 100 µg	-	4.4 ± 3.3	6.6 ± 2.8

Each value is a mean of 3 experiments ± SD.

dust particles obscuring the visualisation of the nuclei (see Figure 4.6). 48 hrs following exposure to DQ_{12}, TiO_2 or Hetton coal, the cells did not possess an increased proportion of multinucleate cells. Exposure to the asbestos samples at the two concentrations for 48 hrs, however, resulted in a significant increase in the occurrence of $P388D_1$ cells containing more than one nucleus ($p<0.05$ between the 20 μg and 100 μg/plate asbestos treated cultures and untreated control value). The large standard deviations around the mean values for the chrysotile treatment at 48 hrs are probably due to the strong cytotoxic capacity of this particular sample.

4.4 DISCUSSION.

The alveolar macrophage is involved in a number of cellular and humoral interactions in the lung, including the phagocytosis of inhaled particulate material; and the recognition of this fact has involved the establishment of extensive studies to examine the direct effect of asbestos fibres on macrophages *in vitro* (reviewed by Miller, 1978). The discovery that chrysotile can modify enzyme loss from cultured macrophages by mechanisms involving either cell death/damage or a selective release of lysosomal enzymes, provided a link between chrysotile inhalation, fibre ingestion by macrophages, and the subsequent induction of localised tissue damage or fibrosis resulting from the release of macrophage hydrolytic enzymes (reviewed by Harington *et al*, 1975; Davies and Allison, 1976; Brain, 1980; Craighead and Mossman, 1982). It was therefore evident that, if the interpretation of the link between the effects of chrysotile fibres on macrophages *in vitro*, and the induction of fibrosis *in vivo* was correct, then i) it would be of great importance to establish why chrysotile has the ability to induce enzyme loss from macrophages and ii) an examination of the effect of asbestos fibres on macrophages may result in the establishment of an *in vitro* assay for the prediction of *in vivo* fibrogenicity. Before establishing such a predictive assay system, however, it was necessary to investigate the apparent inability of the amphiboles to induce a release of macrophage hydrolytic enzymes *in vitro* despite their fibrogenic activity *in vivo* (reviewed by Harington *et al*, 1975). A number of authors have shown that the surface properties of the fibres may be important (Section 1.13.5.3) and more recently it has been demonstrated that fibre length may play an important role in determining the cytotoxic potential of a fibrous sample (Chamberlain *et al*, 1979; Davies, 1980a, b; Davies *et al*, 1980; Beck and Tilkes, 1980; Kaw *et al*, 1982); although Chamberlain *et al* (1979) were the only research workers to show a close correlation between cytotoxic potential towards macrophages and the number of fibres of greater than 10 μm in length. One of the aims of this study was therefore to establish the involvement of fibre length in determining the cytotoxic potential of a fibrous dust sample, in particular with reference to enzyme loss, and the existance of a biologically active fibre length threshold. The $P388D_1$ cell line, a cell line previously

used successfully by other research groups to examine dust/cell interactions (Wade et al, 1976; Gormley et al, 1979) was utilised as a phagocytic substitute for the macrophage in this study, in order to avoid the problems normally associated with the use of macrophage populations (reviewed in Section 4.14). A range of fibrous samples of varying fibre dimensions and fibre numbers was examined (described in Chapter 2 of this thesis), and their abilities to alter cell viability and modify enzyme release from $P388D_1$ cells was established and compared with the characteristic fibre length content of each sample.

4.4.1 The Cytotoxic Abilities of Asbestos Fibres Towards $P388D_1$ Cells - Alteration in Cell Viability and the Relationship With Physicochemical Properties.

The morphological appearance of the $P388D_1$ cells following 48 hrs in culture is shown in Figures 4.1 and 4.5; a mixture of both rounded and spread cells was observed and this finding is in agreement with the descriptions previously reported for these cells (Wade et al, 1976, 1980; Lipkin, 1980). The $P388D_1$ cells were also characteristically macrophage-like in their appearance and their ability to adhere to and spread onto glass; in addition their consituent cytoplasm was highly vacuolated (Figure 4.5) in a manner similar to that reported for activated macrophages (Wing and Remington, 1978). The macrophage-like phagocytic capacity of the $P388D_1$ cells was manifest following their treatment with TiO_2 particles (Figure 4.6), as each cell displayed the ability to clear a particle free "halo" around its circumference. Unfortunately it was not possible to establish the exact number of particles that had been phagocytosed by each $P388D_1$ cell, and it would be expected that a proportion of particles would remain on the external portion of the cell membrane. The occurrence of the ingestion of TiO_2 particles could be established by the examination of TEM sections, but again, this would not allow a quantification of phagocytosis. It was therefore found to be necessary for the purpose of this study, to assume that if a cell had shown the ability to attach a number of TiO_2 particles to its cell membrane, then at least a proportion of these must have been phagocytosed.

Two dust samples TiO_2 (rutile) and DQ_{12} were used as inert and cytotoxic controls respectively in this study (Robock, 1973; Gormley et al, 1979; Harington et al, 1975). All of the cytotoxicity data obtained for the individual dust samples was expressed as the percentage of the value obtained for the non-cytotoxic TiO_2 control in order to remove any effects that were due to the phagocytic event itself, or to inter-experimental variation (Gormley et al, 1979). The morphological appearance of the $P388D_1$ cells following treatment with TiO_2 (Figure 4.6) was consistent with a high viability, and this was reflected in the competent ability of these cells to exclude trypan blue (Table 4.4). It must be noted, however, that after 24 and 48 hrs of treatment with TiO_2, the value for the viability was consistently slightly lower (5 and 15% respectively) than the value obtained for the untreated control; this situation may have occurred because extensive phagocytosis of particles resulted in a slight retardation in the multiplication of the $P388D_1$ cells. Phagocytosis of DQ_{12} , however, resulted in the occurrence of cells which were shrunken and pyknotic in appearance (Figure 4.7), and this observation was confirmed by the low value obtained for the viability of these cells (Table 4.4) after 24 and 48 hrs. The morphological appearance of $P388D_1$ cells was consistent with that reported for macrophages following the phagocytosis of quartz particles (Allison et al, 1966; Harington et al, 1975), and would indicate that the two cell types react in a similar manner. A comparison between the two sets of viability data obtained for the $P388D_1$ cells treated with control dusts (Table 4.4) for 24 and 48 hrs showed similar results ($p>0.05$). This would therefore suggest that, with regard to membrane permeability to trypan blue, the $P388D_1$ cells respond in a similar manner to both TiO_2 and DQ_{12}, even after a period of one year, and thus a direct comparison of results obtained from different dusts examined in different experiments can be made.

The morphological appearance of $P388D_1$ cells following treatment with UICC crocidolite, amosite and chrysotile is shown in Figures 4.2, 4.3 and 4.4 respectively. Wade et al (1976) reported that both crocidolite and amosite had the ability to induce a reduction in the normal pattern of growth curves obtained for $P388D_1$ cells, and a

slight retardation in cell density can be observed for Figures 4.2 and 4.3 compared to the untreated control (Figure 4.1), although the value for cell reduction has not been quantified from the photographs obtained in this study. Wade et al (1976) also showed that chrysotile was cytotoxic towards $P388D_1$ cells, causing a decrease in the growth curve compared to the untreated control, and a number of remaining cells assumed a "sunburst morphology" which was accompanied by an increase in cell size. A similar pattern was observed in this study following treatment of the cells with chrysotile (Figure 4.4) and a considerable reduction in cell density compared to the control (Figure 4.1) was noted . (but not quantified); a number of the cells assumed the "Wade sunburst morphology" and some pyknosis was also observed. The histological appearance of $P388D_1$ cells following ingestion of crocidolite or chrysotile (Figure 4.8 and 4.9) was consistent with the alterations in cell morphology described for animal macrophages following asbestos ingestion (Bey and Harington, 1971; Allison, 1971). The amphibole fibres caused an increased vacuolation of the cytoplasm, and the phagocytosis of chrysotile resulted in considerable pyknosis and cell death. An increase in the number of $P388D_1$ cells containing more than one nucleus was also observed following treatment with amphibole (Figure 4.8) compared to the untreated control (Figure 4.5), This type of result has also been described for $P388D_1$ cells following asbestos ingestion (Lipkin, 1980) and further quantification and discussion of the significance of this finding will be described in a later section of this discussion (Section 4.4.4).

The ability of the various asbestos samples to alter the permeability of the $P388D_1$ cell membrane to the vital dye trypan blue is shown in Table 4.5 and Figures 4.10 and 4.11. This method of assessment of cell viability has been used by a number of research groups (Paul, 1975; Gormley et al, 1979; Pigott and Judge, 1980), and it proved to be simple to use in this study. One disadvantage of the technique is that of subjectivity and also bias of interpretation of results; any effects due to the biased interpretation of the stained cells by an observer were overcome in this study by coding the experimental dishes before they were assessed. A comparison of the results

obtained by different observers (GM Brown, J Whitelaw, A Wright, unpublished observations) showed that the subjective interpretation of the ability of the cells to exclude trypan blue was similar, and that the variation was not greater than 5%.

The general trends shown for the $P388D_1$ cell viability following treatment with the various asbestos samples demonstrated that a concentration of 50 μg/ml of dust was more cytotoxic than 10 μg/ml, and cell viability was higher after 24 hrs than after 48 hrs of exposure to fibres. The viability data was in agreement with the observations regarding the morphological appearance of these cells, in that the UICC chrysotile proved more cytotoxic than either UICC amosite or crocidolite. The general order of cytotoxicity for the different dust types towards $P388D_1$ cells was in agreement with those reports describing the effects of asbestos on primary macrophages, in that the amphiboles proved to be less cytotoxic than the serpentines (Bey and Harington,1971; Kaw and Zaidi, 1975; Harington et al, 1975), and both tremolite (Davies, 1980b) and anthophyllite (Robock and Klosterkötter, 1973) proved more cytotoxic than amosite or crocidolite. The cytotoxic capacity of the various types of fibre may be explained in terms of one, or a combination of a number, of the physicochemical properties of each sample, and these include i) surface properties, ii) particle number or iii) fibre dimensions. Light and Wei (1980) ascribed the biological activity of asbestos samples to the surface properties as manifest by the zeta potential and showed that the order of haemolytic activity for the UICC samples was chrysotile> anthophyllite>amosite>crocidolite (Light and Wei, 1977a). Certainly the order of degree of cytotoxic activity towards $P388D_1$ cells for the 5 UICC samples examined in this study would agree with the sequential order shown by Light and Wei (1977a), although the zeta potential for the fibre samples used in this study was not measured by the author, and it must be considered that the value for each sample may alter considerably during the 24 and 48 hrs culture periods by virtue of the leaching action of the culture medium. Further studies involving the assessment of the alteration of the value of the zeta potential during the leaching of various fibre types by tissue culture medium, and an estimation of the corresponding cytotoxic potential may clarify this situation. Miller and Harington (1972) suggested that the

cytotoxic ability of asbestos was due to the interaction of magnesium ions on the surface of the fibre with the cell membrane glycolipids, thereby causing an increased membrane permeability which would lead to cell death. The order of cytotoxicity obtained for those samples examined in this study would agree with this theory, as the serpentines, which are known to be richly endowed with readily accessible magnesium ions (Section 1.2.1) proved to be more cytotoxic than the amphiboles; in addition the E brucite sample, which is composed of magnesium hydroxide, also proved to be more cytotoxic than the amphiboles (Table 4.5). E H chrysotile (850 °C) consistently proved to be less cytotoxic than the parent E PH sample, and the level of cytotoxicity for this sample was even lower than the value observed for E UICC anthophyllite or E tremolite. This finding was consistent with the report by Hayashi et al (1969) who demonstrated that heating chrysotile to temperatures in excess of 800 °C reduced the surface-active nature of the sample by initiating the rearrangement of the Si-O tetrahedra to form the less reactive forsterite. These types of results have therefore illustrated that there may be a role for the involvement of the surface properties of the fibres in determining the biological activity.

The results obtained regarding the cytotoxic potential of E F amosite and E F chrysotile would suggest that other factors, besides those concerning the surface properties, may be important in determining the cytotoxic potential of an asbestos sample. Both of these samples possessed similar numbers of fibres (ie 58 and 59 fibres/10^{-10}g, respectively), but E F amosite proved to be less cytotoxic than E F chrysotile, thereby illustrating that surface properties may be important. However, E F amosite and chrysotile, whilst possessing relatively fewer fibres than the appropriate UICC asbestos samples (ie 58 rather than 77 fibres/10^{-10}g for the amosite samples, and 59 rather than 185 fibres/10^{-10}g for the chrysotile samples) showed relatively similar degrees of cytotoxicity to their UICC counterparts. This situation could be due to either an alteration in the surface properties of the fibres during their handling in the factory preparation plant, or alternatively, contamination of the samples with material of a cytotoxic nature. The latter hypothesis seems more probable, as some particulate material was found to be contaminating both of

the factory samples (Bolton et al, 1982a), and possibly a further analysis of this material would identify the offending cytotoxic component. This finding illustates that care must be taken when interpreting data from a routine cytotoxicity assay, as the cytotoxic capacity of a sample may not only be due to the fibrous content, but also to the presence of biologically active contaminants.

Elutriation of the UICC samples of crocidolite and amosite did not significantly alter the cytotoxic capacities of these samples. Elutriation of UICC chrysotile, however, resulted in a significant reduction in the cytotoxic capacity of the sample ($p<0.05$), and this may be related to the reduction in fibre number observed following the elutriation process (185 fibres/10^{-10} g for UICC chrysotile and 144 fibres/10^{-10}g for E UICC chrysotile). E ceramic fibre proved to be noticeably "inert" in the $P388D_1$ system, and might be related to the very low constituent fibre number of this sample (7.4 fibres/ 10^{-10} g), which would result in a very low fibre:cell ratio. The cytotoxic capacities of the SF and E LF amosite samples showed some interesting features. The SF amosite sample was prepared originally from the E LF amosite samples, and possessed a greater number of fibres (169 rather than 15 fibres/10^{-10} g), but the SF sample proved to be significantly less cytotoxic than the E LF amosite parent. This finding would suggest that the surface properties of the SF amosite sample had been dramatically altered during the preparation procedure, or alternatively the longer fibres may have a greater cytotoxic potential than the shorter ones. The latter theory would appear appropriate in this case, as a number of research groups have shown similar results upon examination of asbestos samples of different fibre lengths (Davies, 1980a, b; Davies et al, 1980; Kaw et al, 1982).

One of the most cytotoxic of the Group 1 dust samples was the industrially prepared E WDC. This high cytotoxic potential may have been due to either the industrially altered surface properties of the sample, the long and fine nature of the individual fibrils, or the contaminating detergent used during the factory preparation procedure; thus the cytotoxic potential of a number of samples from various stages of the WDC factory preparation procedure were examined in

order to clarify this situation. The results regarding the ability of these WDC samples (Group 2 dusts) to reduce the viability of $P388D_1$ cells are shown in Table 4.15 . It can be seen that the increased cytotoxic potential of the WDC samples was not due to the milling process, because E milled chrysotile showed a similar degree of cytotoxicity to the UICC chrysotile control, and the remaining WDC samples, with the exception of E unextracted WDC, all showed a greater degree of cytotoxicity than E milled chrysotile. This result would suggest that WDC gained its cytotoxic potential during the wet-dipersal process rather than the milling procedure. With regard to the cytotoxic potential of the detergent, the E unextracted WDC sample proved to be the least cytotoxic of the samples examined, whereas the E heat-cleaned WDC showed a high level of cytotoxicity, thereby illustrating that the presence of detergent provided a negligible contribution to the overall cytotoxic ability of the WDC samples, Unfortunately the difficulties encountered in assessing the fibre lengths of WDC did not allow a complete characterisation of any of these samples, and thus it was not possible to relate the cytotoxicity of the WDC samples to fibre dimensions, fibre number or altered surface properties. The problem concerning the identification of the cytotoxic component of WDC can only be overcome by the development of a method which would encourage the complete dispersal of the WDC aggregates, thereby achieving a suspension of individual fibres which could be readily characterised. Should this situation arise, however, it must be considered that the fibre dispersal procedure itself may cause some alteration in fibre surface properties and dimensions, thereby resulting in the production of a sample of fibres which would not necessarily be similar to those airborne fibres available for inhalation by man or animal <u>in vivo</u>; it would appear therefore that some extensive research will be required before this situation is clarified.

In summary, this portion of the study has shown that, with respect to the ability of various fibrous samples to increase the permeability of the cell membrane to trypan blue, the $P388D_1$ cells are more susceptible to the cytotoxic action of serpentine minerals than amphiboles. When considering the physicochemical property of the fibre that determines its cytotoxic potential, it would appear that in some instances the surface properties are involved; although

in the case of SF and LF amosite, the evidence points towards fibre length. A further examination of the involvement of fibre length in determining cytotoxicity is described in Section 4.3.6 of this chapter, and the implications of the results from this study will be described in a later section of this discussion. This portion of the study has described the cytotoxic potential of a number of dust samples in terms of membrane permeability to trypan blue, a number of other cytotoxicity parameters were also measured, including lactate production, and alterations in the levels of the enzymes LDH and glucosaminidase. The modification of these parameters following the treatment of $P388D_1$ cells with dust will be discussed in the following section, and the inter-relationship between cell viability and enzyme release will be examined.

4.4.2 The Relationship Between Cell Viability and Enzyme Levels Following Treatment With Asbestos.

The release of intracellular enzymes from macrophages following the ingestion of mineral dusts has been a topical subject for a number of years (reviewed by Harington *et al*, 1975; Miller, 1978), in particular since the discovery that chrysotile could induce a selective release of lysosomal hydrolases in the absence of any loss of intracellular LDH (Davies et al, 1974a). A number of fibrogenic agents, for example quartz, have been found to cause extensive release of cytoplasmic and lysosomal enzymes (Allison *et al*, 1966), and it has been suggested that these enzymes may be involved in the pathogenesis of fibrosis (Allison, 1971). It is a prerequisite, therefore that an assessment of lysosomal enzyme release should be included in an *in vitro* macrophage cytotoxicity assay, and thus in this study the levels of both cytoplasmic LDH and lysosomal glucosaminidase have been assessed in the $P388D_1$ cell cultures following their treatment with the various dust samples.

The biochemical response of the $P388D_1$ cells following treatment with the control dusts and test dusts is shown in Tables 4.4 and 4.6 to 4.24. The results obtained for both the untreated and dust treated cells have been expressed in a similar manner to the viability data,

ie as a percentage of the result obtained for the TiO_2 treated cultures. The release of lactate following dust treatment was examined in this study because Beck et al (1971) had reported that the treatment of macrophages with a cytotoxic dust involved an increase in output of lactate during cell membrane damage. With regard to the lactate production of $P388D_1$ cells, the untreated and TiO_2 treated cells (Table 4.4) produced similar quantities of this metabolite although a reduction in output was observed following treatment with DQ_{12} for 48 hrs, and this was consistent with the reduction in cell viability induced by this dust. An examination of lactate production by $P388D_1$ cells following treatment with Group 1 and Group 2 dusts (Table 4.6 and 4.16) did not prove useful, however, as the WDC samples were the only dust types which showed the ability to induce an increase in lactate production, and this situation occurred at the 24 hrs time point only. An increase in lactate production generally results from the occurrence of damage to the mitochondrial enzyme system of the cell, and presumably the WDC samples had the ability to initiate this cellular damage by the 24 hrs time point. The lactate assay used in this study was therefore sensitive enough to detect the damage initiated by the most cytotoxic fibrous samples (ie the WDC samples), but not the less biologically active samples (ie the Group 1 dusts). As the measured lactate levels did not show a close correlation with any of the cytotoxicity parameters examined in this study (data not shown), and as it could be assumed that this assay would only be useful in examination of dust samples of great cytotoxic potential, this particular parameter was not examined in any greater depth.

The assessment of the release of the cytoplasmic enzyme LDH has been used previously by a number of research groups to estimate the degree of cell membrane damage induced by exposure of cells to various dust samples (Davies et al, 1974a; Gormley et al, 1979; Davies, 1980a, b; Kaw et al, 1982), and the assay system was therefore incorporated into this study. A comparison of the intracellular levels of LDH for untreated and TiO_2 treated $P388D_1$ cells (Table 4.4) showed a level of intracellular LDH which was consistent with the observed high viability, as assessed by trypan blue exclusion, and this agreed with Davies (1980b) who showed that TiO_2 treatment of macrophages did not result

in a loss of intracellular LDH. DQ_{12} treatment, however, resulted in a reduction of intracellular LDH at 24 and 48 hrs time points, which was consistent with the observed loss of viability; this result confirmed the findings of previous reports concerning the membranolytic nature of this dust sample (Harington et al, 1975; Davies, 1980b). The intracellular levels of LDH following treatment of $P388D_1$ cells with Group 1 and Group 2 dusts are shown in Tables 4.7 and 4.17; a close correlation was noted between the reduction in cell viability and the loss of intracellular LDH at 24 and 48 hrs following treatment with 10 or 50 μg/ml of dust (Figures 4.26 to 4.29). Davies et al (1974a) noted a stimulation of intracellular LDH in his macrophage cultures following exposure to UICC chrysotile, but this was not observed in this study (Figure 4.14); the $P388D_1$ cells showed a marked reduction in the levels of intracellular LDH thereby indicating an increase in membrane permeability. This result suggests, therefore, that the $P388D_1$ cell membrane may show a greater susceptibility to the cytotoxic action of dusts than animal macrophages, and agrees with Daniel and Le Bouffant (1980) who showed that $P388D_1$ cells were more sensitive indicators of dust cytotoxicity than primary macrophages. It did not prove possible to interpret the data concerning the quantity of LDH present in the cell culture fluid or the overall percentage of LDH released per plate (Tables 4.9, 4.10, 4.19 and 4.20), because of the large standard deviations around the means for these parameters. This situation arose because of the extremely low level of LDH released by the $P388D_1$ cells following treatment with the non-cytotoxic TiO_2 particulate control dust. Gormley and Addison (1983) overcame this problem by presenting the data for these two parameters in terms of their absolute values. In this study, all of the data was expressed as a percentage of the TiO_2 control dust in order to remove any inter-experimental variation and also to remove any effects that had been caused purely by the phagocytosis of an inert dust. Gormley and Addison (1983) have presented the remaining portion of their LDH data as a percentage of the TiO_2 control, thereby resulting in an inconsistency in the manner in which they present their results. The values obtained concerning the total levels of LDH per plate did not vary significantly from 100% of the control value, thus demonstrating that LDH was not inactivated after release into the medium, and confirms that phagocytosis of fibrous particles by $P388D_1$ cells does not result in an intracellular

stimulation of LDH. It can reasonably be assumed, therefore, that the interaction of a cytotoxic dust with $P388D_1$ cells results in the release of a quantity of LDH which is adequately reflected by the observed intracellular levels of LDH, thereby negating the necessity to consider the other measured LDH parameters. It was therefore decided that only the intracellular levels of LDH would be examined further.

The release of the lysosomal enzyme n-acetyl-β-D-glucosaminidase was also used as a marker for cytotoxicity in the $P388D_1$ cell assay, and, as described previously, all of the levels were expressed as a percentage of the value obtained for the TiO_2 treated control cells. An examination of the intracellular levels of glucosaminidase following treatment with the control dusts is described in Table 4.4; the $P388D_1$ cells responded to the TiO_2 treatment by showing a rapid loss of intracellular enzyme, and a similar effect was seen for the DQ_{12} treated cells. As the TiO_2 dust sample proved to be non-cytotoxic and $P388D_1$ cells treated with this dust displayed a high viability, the loss of intracellular glucosaminidase can only be interpreted as a selective release of lysosomal enzyme. Davies (1980b) examined the effect of rutile TiO_2 on macrophages, and found no evidence for the induction of this type of selective release, thereby illustrating another difference between the response of $P388D_1$ cells and macrophages to treatment with the same dust sample. A loss of glucosaminidase following treatment of $P388D_1$ cells with DQ_{12} was noted, this has also been reported previously for macrophages (Davies, 1980b), and is consistent with the loss in viability of these cells. The trend in loss of intracellular glucosaminidase following treatment with Group 1 and Group 2 dust samples proved to be similar to the trends noted for the reduction in viability and intracellular LDH levels (Figures 4.26 to 4.29), thus as the cytotoxic capacity of the dust samples increased, the viability, intracellular levels of LDH and glucosaminidase also decreased at a similar rate at each dust concentration and time point. A number of reports have suggested that chrysotile has the capacity to induce a loss of intracellular glucosaminidase in the absence of any loss of LDH from cultured macrophages (Davies *et al*, 1974a; Jaurand *et al*, 1980a; Morgan and Allison, 1980). No evidence for this type of selective release was observed following

the treatment of $P388D_1$ cells, however, and this has revealed another difference in the response of $P388D_1$ cells and macrophages following dust treatment.

The interpretation of the value obtained for the total glucosaminidase levels, the quantity of glucosaminidase in the medium, and the overall percentage of glucosaminidase released per plate, for the $P388D_1$ cells following treatment with the various dust samples proved confusing. The total levels of glucosaminidase per plate proved to be higher for the untreated $P388D_1$ cells than for those cells exposed to TiO_2 (Table 4.4). An examination of the levels following treatment with Group 1 and Group 2 dusts (Tables 4.12 and 4.22) displayed an overall reduction in the total levels following treatment with the more cytotoxic dusts, whereas treatment with a dust of a relatively low cytotoxic potential resulted in a total glucosaminidase level that was similar to the value obtained for the untreated control. This reduction in the glucosaminidase levels can only be explained in terms of "inactivation" of the enzyme upon release from the lysosome into the culture medium. "Activated" macrophages release a variety of proteases (Nathan, 1980), and the possiblity that $P388D_1$ cells may release a protease capable of inactivating glucosaminidase must be considered. Davies et al (1974a) observed a significant increase in the total levels of glucosaminidase per plate following treatment of macrophages with a concentration of 10 μg/ml of UICC chrysotile compared to control cultures; however, the macrophages used in Davies et al's study were not "activated", but had been elicited following intraperitoneal injection of proteose peptone; it is known that protease secretion by macrophages increases upon activation of these cells (Nathan, 1980) and thus the activational status of the macrophages may not have been sufficiently high to allow the secretion of proteases. $P388D_1$ cells have a morphological appearance consistent with cellular activation (Figure 4.5), and are known to have the capacity to release neutral proteases (Werb et al, 1978), although the complete range of proteases produced by these cells has not yet been characterised, and presumably further research will clarify this situation. A close correlation was noted for the relationship between viability, intracellular LDH and glucosaminidase levels, and also the total glucosaminidase levels (Figures 4.26 to 4.29),

thereby suggesting that the glucosaminidase enzyme was inactivated at a constant rate during the induction of cellular damage and subsequent release of internal enzymes. A comparison of the relationship between the overall percentage of glucosaminidase released into the medium and the other cytotoxicity parameters (Figures 4.26 to 4.29) demonstrated that exposure of $P388D_1$ cells to dusts of increasing cytotoxic capacity resulted in an increased cell membrane permeability to trypan blue and also intracellular LDH, and an increased release of lysosomal glucosaminidase. A comparison of the "quantity of glucosaminidase present in the medium" with the other cytotoxicity parameters did not prove useful, however, as no relationships could be seen; this situation was probably complicated by the inactivation of the enzyme upon release into the medium.

In summary, therefore, it was found in this study that the cytotoxic potential of various fibrous dust samples towards $P388D_1$ cells could readily be assessed by measurement of the increase in membrane permeability to trypan blue, the increase in membrane permeability to cytoplasmic LDH, and the increase in loss of lysosomal glucosaminidase. The correlation coefficients for the associations between these assay systems was adequately significant ($p<0.001$) to allow the suggestion that only one of these cytotoxicity parameters would be required in a routine cytotoxicity assay. The lysosomal enzyme was examined in this study in order to establish the existence of selective release by $P388D_1$ cells following treatment with chrysotile. As this result was not obtained and in addition the glucosaminidase data proved difficult to interpret because of the inactivation of this enzyme, it is probable that the use of either the trypan blue exclusion or the LDH release assay would prove the most useful.

4.4.3 The Relationship Between Cell Viability, Enzyme Release and Fibre Length.

A number of recent reports have suggested that fibre length may determine the degree of *in vitro* cytotoxicity exerted by a fibrous dust sample. One of the main aims of this study was to establish the relationship between the cytotoxic potential of a fibrous sample and its ability to modify the viability and enzyme release pattern

of $P388D_1$ cells *in vitro*. A number of the samples examined in the $P388D_1$ cytotoxicity assay did not comply with the appropriate requirements for inclusion in the final portion of this study because of their contamination with non-fibrous material (eg E PH and E H chrysotile, E F amosite and chrysotile), or their inability to disperse adequately in suspension (ie the WDC samples). 13 fibrous samples were therefore selected because of their purity for comparison of cytotoxic capacity with fibre length. A comparison of the relationship between the measured cytotoxicity paramters was undertaken to ensure that these had not altered by virtue of the isolation of this sub-set of data from the main body (Figures 4.30 to 4.33). In general, the trends for this small group of dusts proved similar to those described previously, in that a decrease in viability, and an increase in loss of intracellular LDH and glucosaminidase all corresponded closely to each other and with increasing dust cytotoxicity ($p<0.05$) at both concentrations of dust and at both time points examined, with the exception of the 10 μg/ml concentration at 24 hrs. At this time point and dust concentration the relationship between viability, LDH and glucosaminidase levels was very poor. A comparison of viability and LDH measurements (Figure 4.34) indicated that the viability assay was more sensitive than the LDH assay. It is possible that the exposure of $P388D_1$ cells to dust with a relatively low level of cytotoxicity at 10 μg/ml would result in a very small degree of membrane damage; as the size of the trypan blue molecule is considerably smaller than the LDH molecule ie trypan blue has a molecular weight of 960.8 compared to 140,000 for LDH (Merck Index, 1968; Sober, 1968), it would be expected that a slightly damaged cell membrane would be more permeable to trypan blue than LDH. This effect would have been masked in the previous statistical comparison of the cytotoxicity data, because of the large contribution and therefore bias provided by the very cytotoxic WDC samples. A comparison of viability versus intracellular glucosaminidase (Figure 4.35) and percentage glucosaminidase release (Figure 4.36) did not provide an answer to the apparently poor correlation for the associations between these parameters.

The cytotoxicity data for the 13 fibrous samples was compared with the fibre length data according the the method of Chamberlain *et al* (1979), in which the associations between each cytotoxicity

parameter and the number of fibres that were present in each fibre size range were established (Tables 4.25 to 4.28). Chamberlain et al (1979) showed that those fibres greater than 10 μm in length were the most active towards macrophages in vitro, however the results from the majority of the parameters examined in this study would suggest that those fibres of greater than 8 μm in length were the most active towards $P388D_1$ cells. The results from the trypan blue assay showed that those fibres of greater than 8 μm in length were the most effective towards reducing cell viability at both dust concentrations and time points, and this result agrees with the reports by Wade et al (1980) and Lipkin (1980). These authors showed a close correlation between the ability of various samples of Dawsonite to reduce the rate of growth of $P388D_1$ cells and the number of fibres in the critical size range of greater than 8 μm in length in each sample. The picture proved more complex however, for the relationship between fibre length and the remaining cytotoxicity parameters measured in this study. The decrease in intracellular LDH levels showed a correlation with fibres longer than 20 μm following 24 hrs exposure to 10 μg/ml of dust, 10 μm at 50 μg/ml for 24 hrs, and settled at 7 and 8 μm at 48 hrs for both dust concentrations. The results obtained at 48 hrs for LDH are consistent with the threshold length of 8 μm required for reduction in cell viability, but the LDH assay proved less sensitive at 24 hrs. It has already been demonstrated that the LDH assay is less sensitive than the trypan blue following 24 hrs of exposure of the cells to a low fibre concentration of 10 μg/ml, and it is therefore conceivable that a different fibre length threshold for biological activity would be recorded. The 10 μm length threshold noted following 24 hrs of exposure of the cells to 50 μg/ml of fibres is consistent with the findings of Chamberlain et al (1979) who also used LDH as one of their cytotoxicity parameters. However, in this study the results would suggest that LDH is not the most reliable cytotoxicity parameter to use for the examination of membrane damage, and possibly the use of trypan blue or a more sensitive indicator of membrane permeability (for example ion sensitive dyes) would be recommended.

The interpretation of the comparisons of fibre length with glucosaminidase levels also proved confusing. The total levels of

glucosaminidase corresponded favourably with those fibres of greater than 8 μm in length, in agreement with the viability assessments; the intracellular levels and overall percentage of glucosaminidase released, however, showed a maximum degree of correlation with fibres of greater than 1 or 2 μm in length for the 10 μg/ml concentration at both time points, and 7 and 8 μm for the 50 μg/ml concentration at both time points. This result would indicate that, at a high dust concentration, those fibres that are longer than 8 μm exert a cytotoxic response, thereby resulting in the release of intracellular glucosaminidase into the medium because of cell death. At the lower dust concentration, however, a lower number of fibres of greater than 7 or 8 μm in length were available for cytotoxic interactions with the $P388D_1$ cells, thereby allowing those phagocytosed fibres of greater than 1 or 2 μm in length to exert an effect. Presumably the release of glucosaminidase induced by these smaller fibres was not necessarily a cytotoxic effect, but may have been due to a "selective release" type of enzyme release. The existence of two fibre length thresholds for the induction of lysosomal enzyme release has not been reported previously, but the majority of studies have not been designed in a manner that would result in the detection of this situation. It must be stressed, however, that the interpretation of these findings is purely conjectural, and cannot be substantiated accurately because of the constant inactivation of the glucosaminidase enzyme upon entry into the medium. It is probable that a further study to establish the rate of release of a more stable enzyme may help to clarify this situation; the examination of a variety of dust concentrations ranging between 10 and 50 μg/ml may assist in identifying the fibre concentration at which the 8 μm length fibres start to induce glucosaminidase release by exerting a cytotoxic effect on $P388D_1$ cells.

In summary these studies have shown that, in agreement with previous reports, the longer fibres possess a greater cytotoxic potential than shorter ones (Chamberlain et al, 1979; Davies, 1980a, b; Kaw et al, 1982). The exposure of $P388D_1$ cells to 10 and 50 μg/ml for 24 and 48 hrs resulted in the induction of cell membrane damage by those fibres longer than 8 μm in each dust population. After 24 hrs however, the membrane damage was only sufficient, in some instances, to allow the passage of the trypan blue molecule across the membrane

but not the LDH molecule; only those fibres of greater than 20 μm and 10 μm in length at the 10 and 50 μg/ml concentrations respectively, had the ability to induce enough membrane damage to allow the passage of LDH across the membrane. This result indicates, therefore, that the trypan blue assay is more sensitive with regard to the detection of membrane damage than the LDH assay. Estimations of glucosaminidase release showed that those fibres of over 8 μm in length exerted an overwhelming cytotoxic effect at the higher concentration of dust, and this agrees with the viability and LDH assays; at the lower dust concentration, however, those fibres of greater than 2 μm in length exerted a release of glucosaminidase which may not necessarily be linked to cell death. The possibility that the shorter fibres may have the ability to induce a release of intracellular enzymes is in contrast to the previous reports which link long fibres with *in vitro* pathogenicity, and suggests that they should not necessarily be excluded from consideration.

4.4.4 The Ability of Asbestos Fibres to Increase the Occurrence of Cells With More Than One Nucleus.

A number of previous reports have shown that the exposure of cells to asbestos fibres may result, not only in a reduction in cell viability and an increase in enzyme release, but also in the occurrence of viable cells containing more than one nucleus. This cell syncitium formation has been shown to occur *in vivo* following treatment of rat peritoneal macrophages with asbestos (Sethi *et al*, 1974), and *in vitro* in mouse peritoneal macrophages (Allison, 1971), Green monkey kidney cells (Sethi *et al*, 1974), fibroblasts (Richards and Jacoby, 1976), A549 cells (Chamberlain and Brown, 1978) and $P388D_1$ cells (Lipkin, 1980). The results obtained form this study (Table 4.30) agree with these reports, in that asbestos fibres have the ability to increase the occurrence of cells containing more than one nucleus. It is interesting to note, however, that the occurrence of multinucleate cells was associated only with the ingestion of fibrous dust samples, and not particulate material such as coal. Allison (1971) suggested that giant cell formation may occur when two cells try to ingest the same asbestos fibre, fusion occurring at the point of cell-cell contact. This theory was supported by Sethi *et al* (1974) who, using autoradiographic

labelling techniques, demonstrated that the multinucleate cell formation was due to cell fusion and not inhibition of cell division.

The formation of macrophage polykarya has been found in inflammatory sites during silicosis, asbestosis, sites of lipid deposition and foreign body granulomas (see review by Papadimitriou and Walters, 1979). The mechanism by which cell fusion occurs has still not been elucidated although a number of factors have been associated with increased giant cell formation; these factors include a decreased plasmalemmal calcium concentration (Poste and Reeve, 1972), lysosomal hydrolases (Poste, 1970), decreased intracellular cyclic AMP (Papadimitriou and Sfórcina, 1975) and the presence of a lymphokine-related macrophage fusion factor (Gallindo et al, 1974). The formation of multinucleate giant cells has also been associated with an alteration in the levels of cellular lipid (Papadimitriou and Wyche, 1976), and it has been suggested that a release of lysolecithin may modulate a number of changes in the plasma membrane, thereby facilitating cell fusion (Howell and Lucy, 1969). A number of workers have reported an alteration in cell lipid levels following ingestion of dust (Miller and Harington, 1972; Munder et al, 1966, 1967), and whilst these have always been linked to an increase in membrane permeability and therefore cell death, it is possible that a similar but less traumatic increase in membrane fluidity may encourage cell fusion rather than cell death. It is evident that further work is necessary to determine why fibres are associated with cell fusion, and a further study designed to investigate the pattern of macrophage fusion in vitro and in vivo following treatment with different dust samples, together with an examination of associated biochemical alterations, may serve to elucidate the mechanism of cell fusion during the inflammatory response.

4.4.5 The Usefulness of the P388D$_1$ Cell Assay System - A Comparison With Other Predictive Assay Systems and In Vivo Carcinogenicity.

The P388D$_1$ cell assay system examined in this study proved to be simple to use, and it was possible to obtain an answer with regard to the cytotoxic potential of a dust sample within 3 days. The cell had a number of macrophage-like characteristics which served to recommend its use , and the cells proved similar to macrophages in

their overall response to treatment with dust. One major difference was that chrysotile did not appear to induce the selective release from macrophages previously reported for primary macrophages (Davies et al, 1974a; Harington et al, 1975). The $P388D_1$ cell is not, however, a macrophage but rather a macrophage-like cell, and it is feasible therefore that this cell line would show a slightly different response to that observed for macrophages. As "selective release" of lysosomal enzyme was not observed in this assay system following treatment with chrysotile, the continued study of lysosomal enzyme levels must be questioned. The assessment of membrane permeability would therefore appear to be an adequate method of assessing cytotoxicity, and these studies have shown that, in this case, the trypan blue exclusion test has a greater degree of sensitivity than the LDH assay, and could therefore be recommended for routine use. Gormley et al (1978) and Wright and Gormley (1980) have shown that the direct assessment of cell membrane electrophysiology during the treatment of $P388D_1$ cells with dust samples is an accurate and sensitive method of detecting an increase in cell membrane permeability due to cell membrane damage, and further analysis of this system may result in the development of an assay system that would be even more sensitive than trypan blue exclusion.

A number of in vitro assay systems have been described which reputedly have the ability to predict the potential in vivo fibrogenic and/or tumourigenic capacity of mineral dusts, and these include the haemolysis assay (reviewed in Chapter 3), the A549/V79-4 cell system (Chamberlain et al, 1980), the macrophage (Chamberlain et al, 1979) and the $P388D_1$ cell system (Wade et al, 1980). More recently the haemolysis assay has lost favour because of its lack of correlation with experimental in vivo effects in the lung (Richards et al, 1980). It was concluded, however, at the end of an international meeting concerning the In Vitro Effects of Mineral Dusts (Brown et al, 1980) that the macrophage system may prove useful for predicting fibrogenicity, and the A549, V79-4 and $P388D_1$ systems for predicting tumourigenicity, although it was noted that all of these assays required further investigation and also comparison with in vivo data. Wade et al (1980) showed that the cytotoxic activity of fibrous samples towards $P388D_1$ cells corresponded to the availability of fibres of greater

than 8 μm in length, and this was in agreement with the Stanton Hypothesis regarding *in vivo* carcinogenicity and fibre length. The results from this study agreed with Wade *et al* (1980), as the cytotoxic potentials of the various asbestos samples examined showed a close relationship with the number of fibres of greater than 8 μm in length; this finding also concurred with that of Chamberlain *et al* (1980) who surmised, as a result of their *in vitro* experiments, that fibres of greater than 8 μm in length would prove pathogenic *in vivo*.

A comparison of the response of $P388D_1$ cells, V79-4 and A549 cells to treatment with various WDC samples showed that the three cell types gave a similar result with regard to the order of cytotoxic potential of these particular dusts (Gormley *et al*, in press). The results from these three tests indicated that the WDC samples would prove tumourigenic, and a comparison with preliminary *in vivo* data showed that all of the dusts had the ability to induce mesotheliomas, although the order of degree of carcinogenicity did not entirely agree with the order predicted by the *in vitro* tests. An earlier study, however, showed a close correlation between the carcinogenic potential of a number of asbestos samples and their cytotoxic activity towards $P388D_1$ cells *in vitro* (Bolton *et al*, 1982a), thereby indicating the potential usefulness of this *in vitro* model. One disadvantage of the $P388D_1$ system is its inability to distinguish between the potential fibrogenic and/or carcinogenic capacity of a dust sample, and further work is required to satisfy this need, possibly making use of the observation that only fibrous dust samples initiate the increased occurrence of multinucleate cells. A number of studies are currently underway at the IOM to investigate further the predictive nature of the $P388D_1$, A549 and V79-4 cell systems, and the corresponding *in vivo* data will soon be available for comparison. It is hoped that these studies will contribute considerably towards clarifying the relationship between fibre length, *in vitro* cytotoxicity and the *in vivo* pathogenicity of asbestos samples.

4.4.6 CONCLUSIONS.

This study comprised an examination of the response of $P388D_1$ cells to treatment with various fibrous samples *in vitro*, in particular with regard to an increase in membrane permeability and lysosomal enzyme release. In general, the cells reacted in a similar manner to the response described for primary macrophages, although no evidence for a selective release of lysosomal enzyme following treatment with chrysotile was noted. A group of fibrous samples prepared by the industrial wet-dipersal process proved to be particularly cytolytic in nature. There was a close relationship between the cytotoxic potential of the asbestos samples and an increase in cell membrane permeability to trypan blue and LDH, and a release of lysosomal glucosaminidase; the degree of cytotoxicity was shown to be related to the number of fibres of greater than 8 μm in length present in each sample. A comparison of the $P388D_1$ system with other predictive assays and preliminary *in vivo* data would indicate that the $P388D_1$ system may be useful as an *in vitro* predictive model.

CHAPTER 5 ASBESTOS-INDUCED ALTERATIONS IN THE P388D$_1$ CELL MEMBRANE ELECTROPHYSIOLOGY.

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CHAPTER 5 ASBESTOS-INDUCED ALTERATIONS IN THE P388D$_1$ CELL MEMBRANE ELECTROPHYSIOLOGY.

5.1 INTRODUCTION.

The macrophage is actively involved in a number of processes in the body including phagocytosis (Reynolds et al, 1975), secretion (Davies and Bonney, 1980), chemotaxis (Wilkinson and Allan, 1980), destruction of microbes and tumour cells (Nathan et al, 1982) and mediation between various components of the immunological system of the host (Unanue,1980). In order that these functions be carried out efficiently, it is generally a prerequisite that a direct interaction with the cell plasma membrane via receptors must occur. Phagocytosis is often mediated via Fc, C3 and other membrane receptors (Reynolds et al, 1975; Weir and Ogmundsdöttir, 1977; Benoliel et al, 1980); enzyme secretion is often enhanced upon the attachment and phagocytosis of various particles (Davies and Bonney, 1980). Chemotaxis following macrophage stimulation by N-formylated peptides requires the recognition of the chemoattractant by a specific cell-surface receptor (Snyderman and Fudman, 1980); destruction of microbes also necessitates interaction with the cell membrane (Leijh et al, 1980), and immunological mediation often requires the involvement of the plasma membrane via phagocytosis and antigen presentation (Unanue, 1980). It can be demonstrated therefore that the macrophage membrane plays a considerable role in the expression of these functions, and it would be expected that some perturbation of the membrane integrity may occur when the cell embarks on one or more of these interactions. In order to try and clarify this situation, a number of research workers have recently started to study the electrophysiological properties of the macrophage membrane, as an alteration in these properties may be involved in the initiation or management of each interaction; these studies will be described in the following section.

It should be noted that a brief description of those properties of the cell membrane which contribute to the generation of the various electrical properties may be found in Section 5.1.3.

5.1.1 Interactions Involving Alterations in the Electrical Properties of the Macrophage Membrane.

The electrophysiological properties of the macrophage membrane have been investigated by a number of research groups. Populations of macrophages from a number of sources which had been elicited *in vivo* by different stimulating agents and cultured *in vitro* for varying periods of time were examined. Gallin *et al* (1975) studied guinea pig macrophages induced following an intra-peritoneal injection of mineral oil; the macrophages were cultured for 4 days prior to examination, and the authors recorded an average transmembrane potential (MP) of -13 millivolts (mV) and input resistance (IR) of 140 megohms (MΩ) for the cell population. It was noted that a proportion of these cells had the ability to exhibit spontaneous hyperpolarisations of approximately -10 to -50 mV in amplitude (Gallin *et al*, 1975). Gallin and Gallin (1977) also examined human peripheral monocytes 3 weeks after culture *in vitro*, and mean MP and IR values of -14.5 mV and 84.4 MΩ respectively were recorded; in addition the occurrence of spontaneous hyperpolarisations were noted for these cells. Dos Reis *et al* (1979) also examined oil-elicited mouse and guinea pig peritoneal macrophages and recorded mean MP values of -26 and -18 mV respectively as well as the occurrence of slow hyperpolarisations. Thioglycollate-elicited mouse peritoneal macrophages were shown to have resting potentials that were generally in the region of -20 to -40 mV, although a small proportion of the populations had high MP values of -60 to -90 mV, and both types of cell exhibited spontaneous hyperpolarisations (Gallin and Livengood, 1980). A number of other research groups have obtained their macrophage populations by implanting discs in the peritoneal cavities of rats or mice; after a period of time the discs may be removed and the adherent macrophages examined. Using this method, Kouri *et al* (1980) showed that rat peritoneal macrophages had a mean MP of -13 mV, and Persechini *et al* (1981) found that mouse and rat polykarya had MPs that ranged from -5 to -40 mV and IR values from 14 to 120 MΩ for mouse cells and 5 to 45 MΩ for rat cells. The macrophage polykarya also exhibited the spontaneous slow membrane hyperpolarisation previously noted for mononuclear cells (Persechini *et al*, 1981).

The occurrence of the slow membrane hyperpolarisation has proven to be of particular interest, as it not only occurs in a spontaneous manner, but has also been noted following the mechanical and electrical stimulation of macrophages (Gallin et al, 1975; Dos Reis et al, 1979), during the phagocytosis of latex beads (Kouri et al, 1980) and during the initial period of exposure of macrophages to the chemotactic factors C5a and synthetic N-formyl methionyl peptide (Gallin and Gallin, 1977). An examination of the hyperpolarisation response has shown that this electrical phenomenon is associated with increased flow of potassium ions (K^+) out of the macrophage (Dos Reis et al, 1979; Oliveira-Castro and Dos Reis, 1981); the introduction of calcium ions into the cell either via direct injection or the use of a calcium ionophore served to sustain the hyperpolarisation response, and the use of a calcium chelator (Mg-EGTA) abolished the effect, thereby illustrating that the response is also sensitive to the level of intracellular and extracellular calcium (Gallin and Gallin, 1977; Gallin et al, 1975; Oliveira-Castro and Dos Reis, 1981; Persechini et al, 1981). These workers have therefore concluded that the macrophage slow hyperpolarisation probably involves the activation of the Ca^{++}-sensitive K^+ conductance channel, and this response may be important in the development of various macrophage interactions including cellular activation. Gallin and Gallin (1977) noted that the macrophage membrane hyperpolarisation response following stimulation by a chemoattractant preceded an increase in membrane spreading, ruffling and pseudopod formation, and Niemtzow et al (1979) observed an increase in the MP value when macrophages were exposed to lymphokine in vitro.

It would appear therefore, that the measurement of the macrophage membrane electrical activity is a sensitive indicator of the initiation of various membrane interactions, and this fact has been recognised and applied to studies of the interaction of mineral particles with macrophages. Gormley et al (1978) compared the response of guinea pig alveolar macrophages and $P388D_1$ macrophage-like cells following treatment with different particulate samples. These cell types were shown to have initial mean MP values of -6.0 and -6.4 mV respectively. After exposure to dust samples, the initial response of each cell type was generally similar in that an initial perturbation of the MP occurred;

treatment with a non-cytotoxic dust resulted in the MP returning to the resting value, whereas the cytotoxic dusts induced a membrane depolarisation which was consistent with cell death. Both cell types exhibited spontaneous hyperpolarisations which increased in frequency upon addition of dust (Gormley et al, 1978). This work was extended to an examination of the effects of the UICC asbestos samples on $P388D_1$ cell membrane electrophysiology (Gormley and Wright, 1980; Wright and Gormley, 1980). The authors found that the cytotoxic chrysotile sample caused a reduction in the MP value which was consistent with a reduced cellular viability, whereas the non-cytotoxic crocidolite had little effect; amosite proved interesting by virtue of its ability to induce a marked membrane hyperpolarisation, although this effect was not investigated further (Gormley and Wright, 1980). These studies have demonstrated that the measurement of cell membrane electrophysiology is a sensitive indicator of membrane damage following treatment with dust, and in particular it can detect unusual events such as the hyperpolarisation induced by UICC amosite. An examination of the effect of complement on cultured nerve and muscle cells has already shown that membrane damage may be detected using electrophysiological techniques approximately 10 to 60 minutes earlier than the trypan blue test (Stephens and Henkart, 1979), thereby illustrating the greater sensitivity of this assay. A further examination of fibre-induced alterations in cell membrane electrophysiology would be of some importance in order to establish the usefulness of this assay for inclusion in a routine cytotoxicity system; in addition an examination of the amosite-induced hyperpolarisation may assist towards clarifying the mechanism of interaction of fibrous particles with the macrophage membrane. The use of the $P388D_1$ cell line would be advantageous in this type of study because of its easy accessibility and its macrophage-like characteristics, it would also overcome the problems associated with the choice of macrophage population and activational state.

5.1.2 Aims and Objectives.

i) To examine the effects of UICC crocidolite, amosite and chrysotile on the MP and IR values for $P388D_1$ cells, in particular with reference to the possible protective role of serum.

ii) To examine the ability of amosite samples from a variety of sources to elicit a membrane hyperpolarisation, and to establish the nature of this response.

iii) To assess the usefulness of this technique as a sensitive indicator of the cytotoxic capacity of a dust sample.

5.1.3 Basic Principles Regarding the Generation and Maintenance of the Cell Membrane Potential and Input Resistance.

This section comprises a brief description of those features of the cell membrane which result in the manifestation of a cell MP and IR. As these features have been described in detail in a number of text-books including Aidley (1971) and Finean et al (1978), only the major conclusions will be described here.

The basic structure of the cell plasma membrane has been described in detail by Singer and Nicolson (1972) and Nicolson (1976a,b), and comprises a lipid/protein bilayer, which may vary in structure depending on the cell type. The main function of the membrane is to separate the intracellular matrix from the extracellular fluid, and it is a complex of phospholipids, the hydrophobic ends of which meet at the membrane interior and the hydrophilic ends are exposed to the extracellular and intracellular environments. The overall fluidity is inversely proportional to the ratio of cholesterol:phospholipid. A number of glycoprotein and lipoprotein structures are found embedded in the membrane (Nicolson, 1976a), these may extend to either edge of the bilayer and some of the glycoproteins on the exterior membrane act as receptors for hormones, antibodies and other stimulating proteins. A proportion of those glycoprotein structures that extend across the whole of the lipid bilayer are attached to cytoskeletal components of the intracellular matrix ie the microtubules and microfilaments; this attachment often allows a direct communication between a number of stimulating agents in the extracellular environment and responding elements in the intracellular complex (Nicolson, 1976b).

Across the cell membrane, concentration differences for various ions are developed. Table 5.1 shows the extracellular and intracellular values for the concentrations of potassium (K^+), sodium (Na^+) and chloride (Cl^-) observed for the frog muscle. The ratios of intracellular: extracellular concentration for each of these ions are typical for many cell types, although the exact ratios may differ upon the type of cell examined. It will be noted that potassium is the main intracellular cation, and sodium is the major cation in the extracellular fluid. Calcium (Ca^{++}) and magnesium (Mg^{++}) ions also differ in concentration

Table 5.1 Ionic Concentration In Frog Muscle Fibres and Plasma.

Ion	Concentration in Muscle Fibre (mM/1)	Concentration in Plasma (mM/1)
K^+	124	2.25
Na^+	10.4	109
Cl^-	1.5	77.5

After Aidley (1971).

across the cell membrane; the intracellular Ca^{++} concentration in the resting state being low (approximately 10^{-7}M). The major intracellular fluid anion is protein, which is negatively charged at intracellular pH.

Various conductance channels are present in the cell membrane which allow the passage of ions eg Na^+, K^+, Ca^{++} etc (Finean et al, 1978; Hagiwara, 1981). A transmembrane potential difference arises across the plasma membrane, the inside of the cell being negatively charged with respect to the cell exterior. This arises as a result of the permeability of the membrane to K^+ being much greater than the permeability to Na^+. More K^+ ions leave the cell than Na^+ ions enter it in a given time, leaving negatively charged proteins in the cell interior which cannot accompany the K^+ out of the cell. The inside of the cell then has a net negative charge and the exterior a net positive charge. Other ions also contribute to the development of a transmembrane potential in proportion to their ionic permeabilities. The magnitude of the membrane potential developed has been described by the Goldman Equation (Figure 5.1) (Goldman, 1943; Hodgkin and Katz, 1949). This equation illustrates the fact that an alteration in the permeability of the membrane to a particular ion will result in an alteration in the MP, even if no gross alteration in intracellular or extracellular ionic concentration is observed.

Sodium/Potassium Pump.

As stated previously, the permeability of the membrane to Na^+ is much smaller than the permeability to K^+, but there is a slow steady leakage of Na^+ into the cell down an electrochemical gradient. This is countered by the Na^+/K^+ pump, which exchanges 3 intracellular Na^+ for 2 extracellular K^+; this system requires energy which is derived from the breakdown of ATP (Finean et al, 1978).

Calcium/Sodium Coupled Pump.

Calcium ions which enter the cell via the calcium conductance channels are removed from the cells by the Ca^{++}/Na^+ coupled pump (Blaustein, 1974). Studies of intracellular calcium levels have

$$E_m = \frac{-RT}{F} \log_{10} \frac{P_K[K^+]_o + P_{Na}[Na^+]_o + P_{Cl}[Cl^-]_i}{P_K[K^+]_i + P_{Na}[Na^+]_i + P_{Cl}[Cl^-]_o} \text{ mV}$$

where: E_m = potential difference across membrane in volts

R = gas constant of 8.31 Joules/degree absolute/mole

F = Faraday constant ie 96,500 coulombs/mole

T = 293 °Absolute (Kelvin)(at 20 °C)

P = membrane permeability coefficient for any one ion

$[\ ]_o$ = extracellular concentration M/l

$[\ ]_i$ = intracellular concentration M/l

Figure 5.1 The Goldman Equation.

demonstrated that Ca^{++} efflux is due to an exchange of internal Ca^{++} and external Na^{+}. In general two Na^{+} are exchanged for one Ca^{+}, and the energy is supplied by the Na^{+} gradient generated by the Na^{+} pump.

Calcium Sensitive Potassium Conductance Channels.

In many cells, for example the erythrocytes, cultured dorsal root ganglion cells, frog muscle fibres and mouse macrophages, K^{+} efflux from the cell via a Ca^{++} sensitive K^{+} conductance channel, leads to a hyperpolarisation of the cell (Gardos et al, 1975; Lew and Ferriera, 1979; Dos Reis et al, 1979). Quinidine and quinine are effective blocking agents of this channel.

Membrane Resistance and Capacitance.

The cell membrane can be drawn in terms of a simple electrical circuit (Figure 5.2) (Aidley, 1971). The cell membrane has capacitance properties because of the role it plays as an imperfect insulator separating 2 conductors ie the ICF and ECF (Figure 5.2). The extracellular and intracellular fluids have a resistance to current flow of r_o and r_i respectively. The membrane by virtue of its property of producing limited permeability to certain ions can be thought of as having a resistance r_m (membrane resistance) ohms/cm, to the passage of ionic current. It may be considered that the opposition encountered by an ion during its passage across the membrane is termed the resistance to the passage of the ion. This resistance to the passage of particular ions is often stated in terms of the conductance to the ion(g) eg for Na^{+}:

$$gNa = \frac{1}{R_{Na}}$$

If the resistance to the passage of Na^{+} ions falls then the conductance of the membrane to Na^{+} rises. The total membrane conductance (g_M) is the sum of the partial ion conductances ie:

$$g_M = K^{+} + Na^{+} + Ca^{++} + Cl^{-} \ldots\ldots\ldots\ldots\ldots$$

Membrane conductance to particular ions is related, in an albeit complex way, to the membrane permeability to that ion. Thus, the measurement of the cell membrane resistance to ionic flow is a sensitive method of assessing the membrane permeability; if the membrane resistance is low, then the ionic flow will increase, thus leading to an alteration in

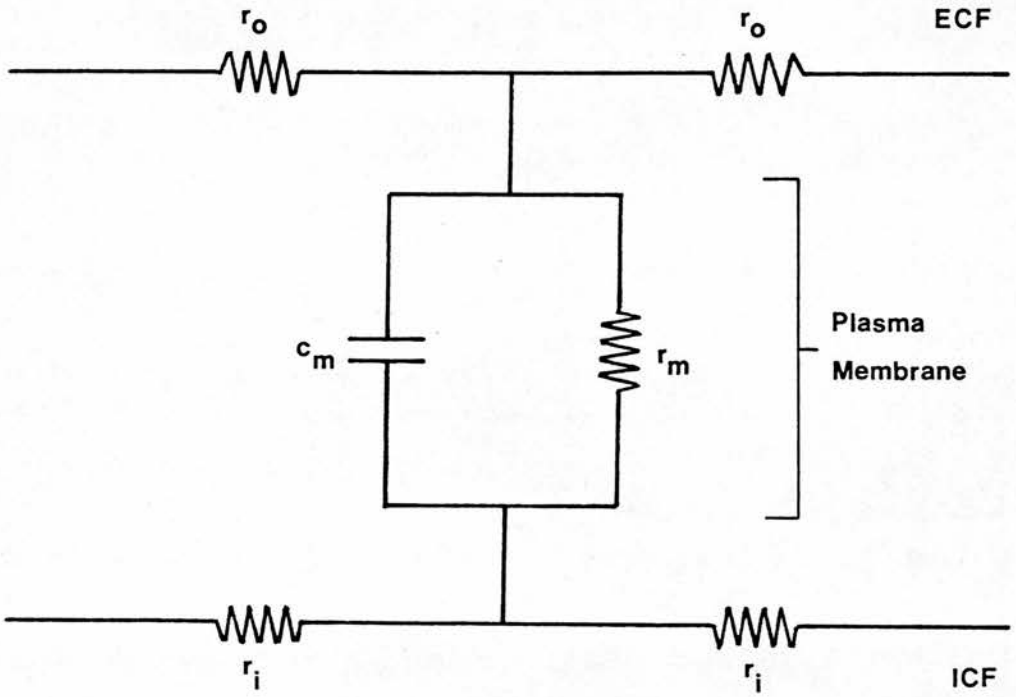


Figure 5.2 Simple Electrical Circuit Simulating the Electrical Structure of a Section of the Cell Membrane.

Where:

ECF = extracellular fluid

ICF = intracellular fluid

c_m = capacitance of membrane/unit length, μF/cm

r_o = resistance of ECF/unit length, ohms/cm

r_i = resistance of ICF/unit length, ohms/cm

r_m = resistance of membrane (inside to outside)/unit length, ohms/cm

the MP. The measurement of MP is a sensitive indicator of the integrity of the cell membrane.

5.1.4 Principles Regarding the Measurement of Cell Membrane Potential and Resistance.

The potential difference across the cell membrane ie the trans-membrane potential can be measured using the apparatus shown in Figure 5.3. Under high power microscopic observation and using a micromanipulator it is possible to insert a microelectrode (ME) across the cell membrane and into the cell (Finean et al, 1978), the MEs are made of glass and have a high resistance of 5 to 50 MΩ and tip diameters of 0.2 to 0.5 μm. The ME is filled with 3 M potassium chloride (KCl) solution to provide electrical contact with the intracellular fluid. A silver wire coated with silver chloride, dipped into the KCl solution, connects the electrode to a high impedance amplifier stage. As the potential difference across the cell membrane is small (less than 100 mV), the voltage signal requires amplification before being viewed on the oscilloscope. In order to quantitatively measure the voltage across a ME when it is in the cell, the recording system must have an input resistance at least 100 times greater than that of the ME. Oscilloscopes have an IR of 2 MΩ and therefore cannot be connected directly to the ME. A high impedance head containing a field effector transistor and integrated circuit amplifier is therefore introduced between the ME and the oscilloscope. The high impedance head has infinite input impedance and this draws no current from the biological system, it is therefore usefully employed as the first stage in a voltage measuring system.

A silver chloride coated silver wire, placed in the periphery of the culture dish, serves as an earth electrode, and is connected to earth via a calibrator box. The MP is measured by recording the voltage change observed on the oscilloscope screen when the ME is inserted into the cell; the calibrator is employed to accurately measure this voltage change. Using a voltage from the calibrator, the outside solution is charged positively relative to the reference input, so the intracellular potential (originally negative relative to earth) now becomes earth relative to the positive outside. This may be observed

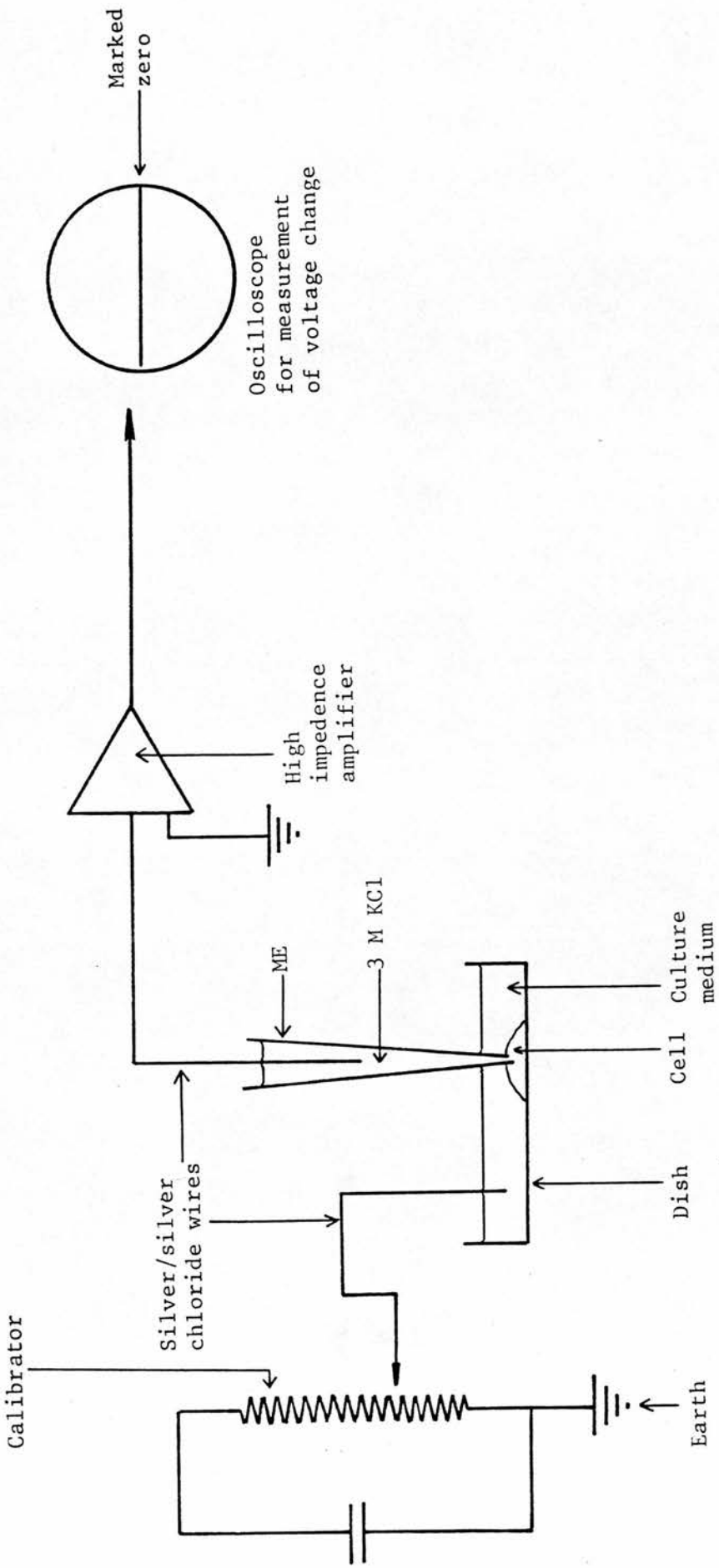


Figure 5.3 Apparatus for Measurement of Membrane Resting Potential.

on the oscilloscope screen when the deflected beam moves step by step towards the original zero position during the application of increasing steps of positive potential from the calibrator (this is termed "backing off").

A number of methods are available for measurement of membrane resistance:

i) Two microelectrodes may be inserted into the cell; a current pulse is passed through one, and the voltage change produced by the pulse is recorded by the other one. Ohm's Law is applied to calculate the input resistance (IR). This method is only useful if two electrodes can be inserted into one cell (Bennett et al, 1959; Hagiwara et al, 1959).

ii) A double barrel ME technique involves the use of two MEs which have been fashioned from 2 glass capillaries forced together. Current is passed through one electrode and voltage measured via the other. The IR is calculated using Ohm's Law (Coombs et al, 1955; Coombs et al, 1959).

Unfortunately methods i) and ii) are limited only to the study of large cells that can withstand the twin impalements. This problem has been overcome by the use of a Wheatstone Bridge technique (Araki and Otani, 1955), which allows the direct stimulation of an impaled cell via the same electrode that is used for recording MP. The principle comprises a four arm Wheatstone Bridge (Figure 5.4) in which one of the 4 arms is the recording ME (R4). The resistances on the bridge are adjusted so that a zero potential difference is observed between the two terminals C and D during the application of a current pulse between terminals A and B. When no potential difference is recorded between C and D, the bridge is "balanced", and at this stage a precise relationship exists between the values for the 4 resistances of the bridge ie:

$$\frac{R1}{R3} = \frac{R2}{R4}$$

When the electrode is placed in the external medium that bathes the cells, the Bridge must be balanced to account for the electrode resistance; the ME resistance can be calculated using the above formula (resistance value I). Upon the insertion of the ME into the cell, there is a change

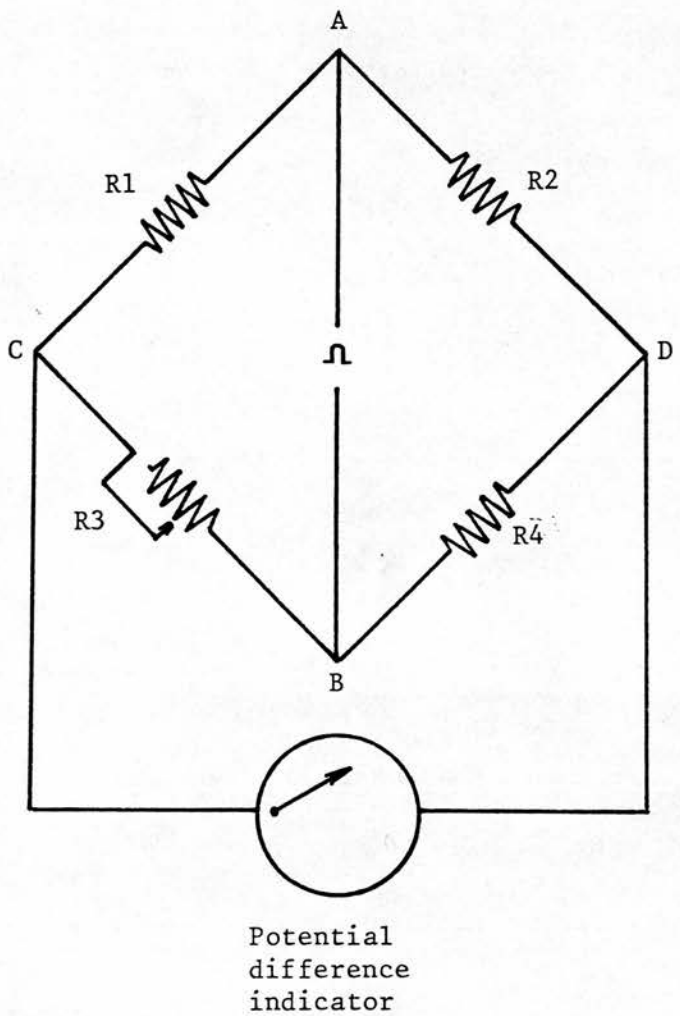


Figure 5.4 The Wheatstone Bridge.

$$\frac{R1}{R3} = \frac{R2}{R4}$$

Where:

R1 and R2 are constant resistances

R3 = variable resistance

R4 = resistance due to ME or ME and cell IR

in the voltage level in a negative direction due to the potential difference across the cell membrane, and the Bridge becomes unbalanced due to the additional resistance of the cell membrane. The Bridge is rebalanced so that a null recording is obtained on the potential difference indicator; the value of R4 due to the electrode resistance plus the resistance of the cell membrane (IR) can then be calculated (resistance value II). The IR can then be calculated by subtracting value I from value II. This method does not account for any alterations in the specific electrode resistance due to entry into the cell, and the method used to account for this alteration will be described in the Materials and Methods sections of this Chapter. The overall cell IR (R_o MΩ) is the sum of the individual resistances for each unit area of cell membrane. For spherical cells such as macrophages it is considered that:

$$R_o = \frac{R_m}{\text{surface area}}$$

where R_m = resistance/unit area of membrane

Unfortunately it is not possible to calculate the R_m values for the macrophage because of the many pseudopodia and ruffles that extend from this cell. The R_o value must therefore be considered a representative measure of the R_m value.

The use and application of the basic principle discussed in this section for the measurement of MP and IR of the $P388D_1$ cell membrane will be described in the Materials and Methods Section of this Chapter.

5.2 MATERIALS AND METHODS.

5.2.1 The Manufacture of Glass Microelectrodes.

Glass ME were manufactured using a Narishige ME Puller Type PE-2. Capillary glass type GC 150F-10 (Clarke Electromedical Instruments) containing a single filament for easy filling was used. The settings for the ME puller heater and magnet were adjusted until MEs were consistently obtained that were of a satisfactory tip resistance (20 to 40 MΩ) and which entered the cell easily. The tip diameters varied between 0.2 to 0.5 μm (checked using the SEM). After pulling, the MEs were allowed to cool, and placed tip downwards in a beaker of 3 M KCl; the electrode tips filled by capillarity. The electrode shafts were filled with 3 M KCl using a syringe and fine bore needle.

5.2.2 The Chloriding of the Silver Wire Electrodes.

The silver wire electrodes used in the experiments were coated with silver chloride before use in order to minimise any "potential drift" and to produce non-polarisable electrodes. The electrodes were cleaned with emery paper prior to coating. Each silver wire electrode in turn was dipped into a beaker containing N/10 HCl, and a plating circuit was created by attaching the electrode to the positive terminal of a 9 V battery (Ever Ready PP7). A second electroplating electrode, also of silver, was made the cathode. The current was allowed to flow for 30 secs and after this time the current was reversed. This cycle was repeated 3 times until the non-polarisable electrode was uniformly coated with a layer of silver chloride.

5.2.3 The Equipment Used and the Procedure Employed for the Measurement of Cell Membrane Potential and Input Resistance.

The apparatus used for the measurement of MP and IR is shown diagrammatically in Figure 5.5. The circuit diagram of the main box containing ME amplifier and Wheatstone Bridge is shown in Figure 5.6. The micromanipulator and microscope were mounted on a metal box containing 1.5 cwts of concrete, and the box was mounted on three mushroom shock absorbers. This structure greatly enhanced the stability of the

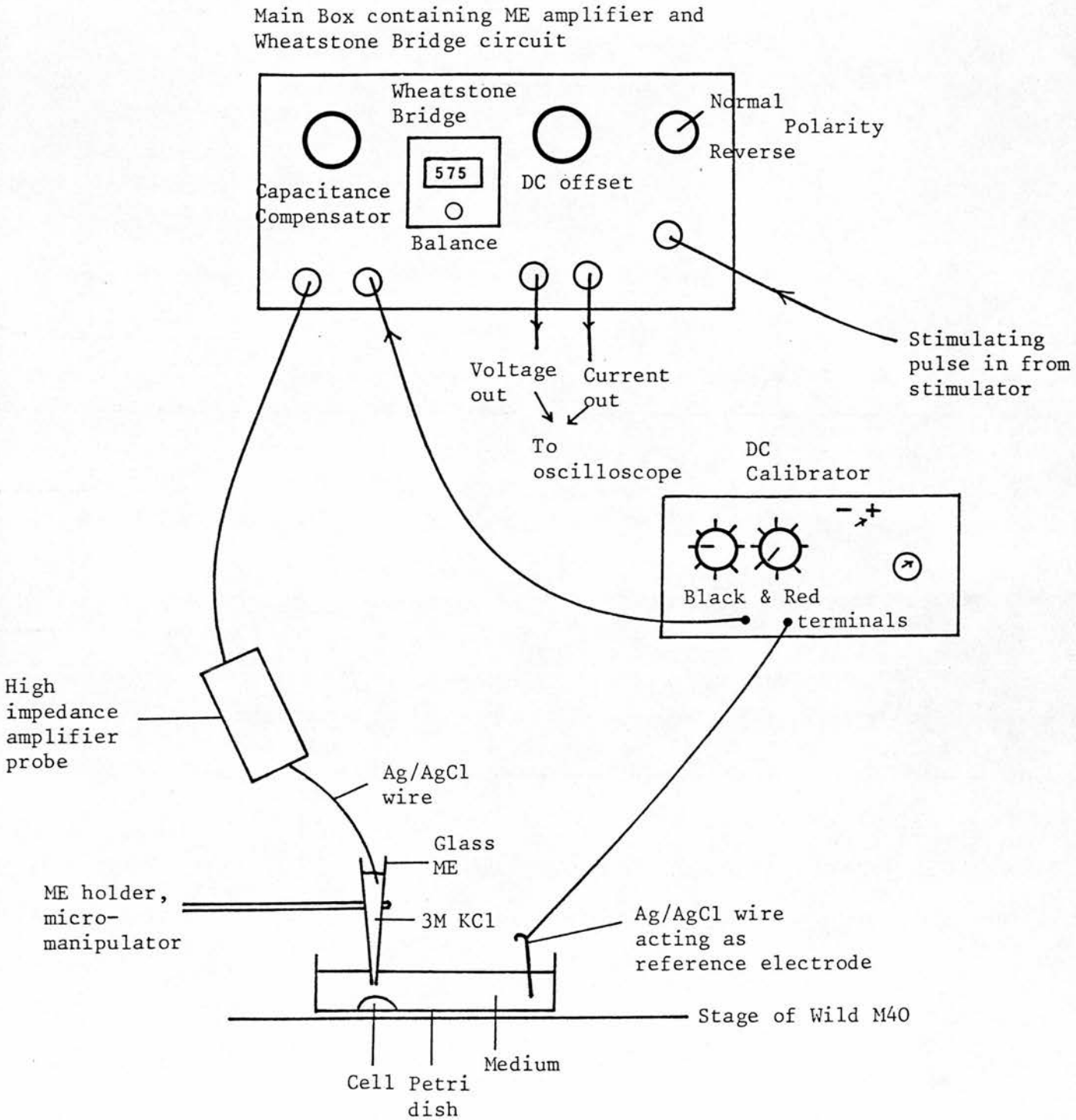


Figure 5.5 Apparatus Used for Measurement of Cell Membrane Potential and Input Resistance.

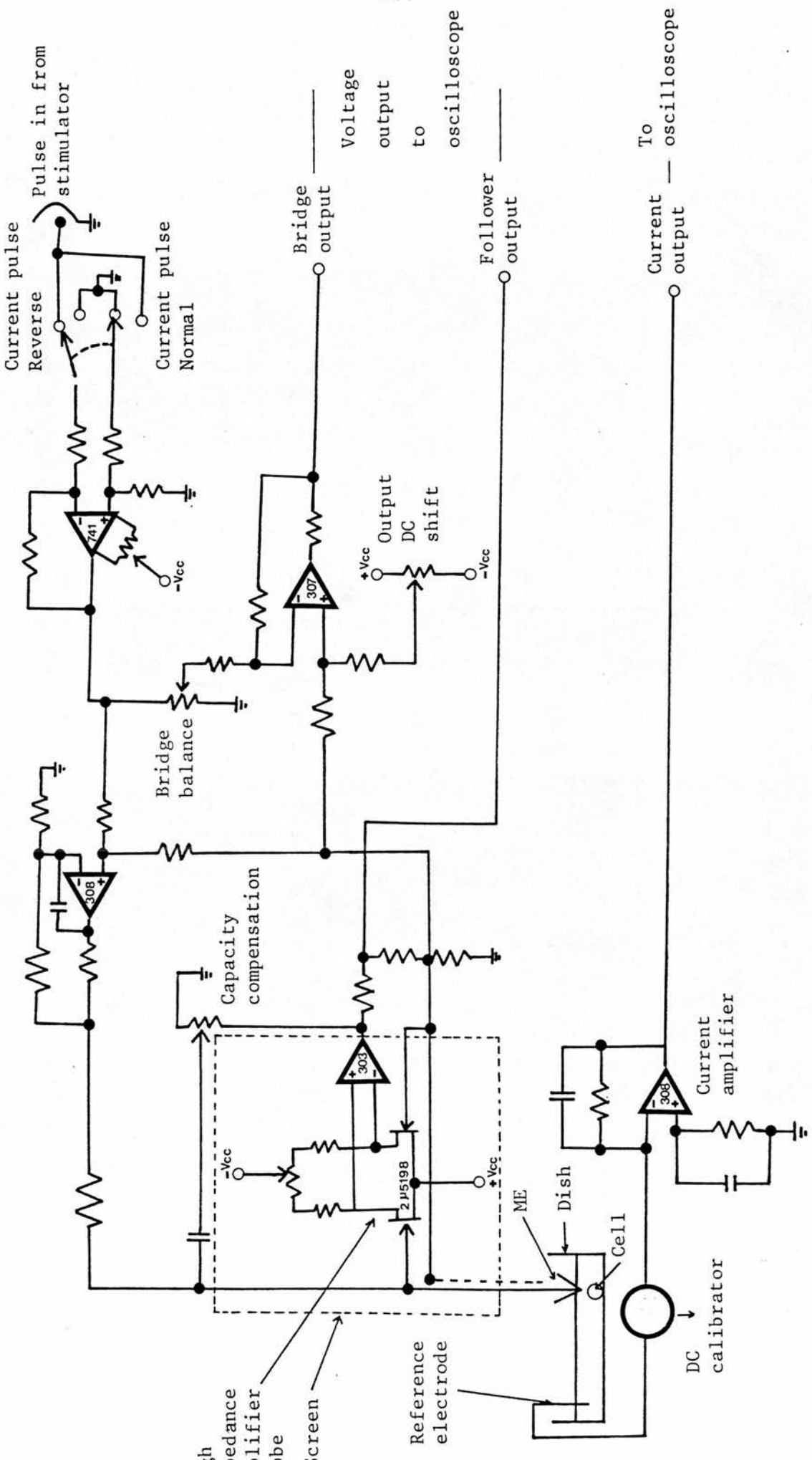


Figure 5.6 Circuit Diagram for Main Box Containing ME Amplifier and Wheatstone Bridge.

apparatus, and was required if a ME was to be inserted into the cells accurately. The inverted microscope, ME and high impedance amplifier probe were placed inside a wire cage; the cage was earthed so that any extraneous electromagnetic or induced currents would pass to earth and not interfere with the measurements of the MP.

The following procedure was employed for measuring the cell MP and IR:

1) A culture dish of P388D$_1$ cells was removed from the incubator and placed on the stage of the Wild M40 Inverted Microscope.

2) A freshly chlorided silver wire reference electrode was attached to the red terminal of the DC calibrator and placed in the culture medium at the periphery of the culture dish.

3) The black terminal of the DC calibrator was connected to the effective earth voltage point of the main box.

4) A glass ME was placed in a ME holder attached to a Zeiss micromanipulator; the ME was lowered, using the micromanipulator, until the tip was just below the surface of the culture medium.

5) The chlorided silver wire input lead was inserted into the KCl solution in the ME and connected to the input of a high impedance amplifier probe. The output of the probe was connected to the main box.

6) The voltage and current outputs of the main box were connected to the oscilloscope (Gould Advance Digital Storage Oscilloscope, Type OS 4000) amplifier inputs. A lead from the stimulator was connected to the current pulse input of the main box.

7) The stimulator and oscilloscope were switched on.

8) An audio unit (voltage dependent oscillator) was switched on. The unit emitted an audible note, the frequency of which was proportional to the position of the voltage beam on the oscilloscope screen. This was utilised in order that the observer could maintain visual observation of the insertion of the ME into the cell. The entry of the ME into the cell was signalled by a change in the frequency of the note as the oscilloscope beam descended the oscilloscope face during the recording of the MP.

9) A "zero-point" base-line was established on the oscilloscope screen by switching the voltage channel input to "ground", and then moving the voltage trace with the "shift control" of the oscilloscope. The "gain" control was set to 10 mV/cm to calibrate the oscilloscope screen.

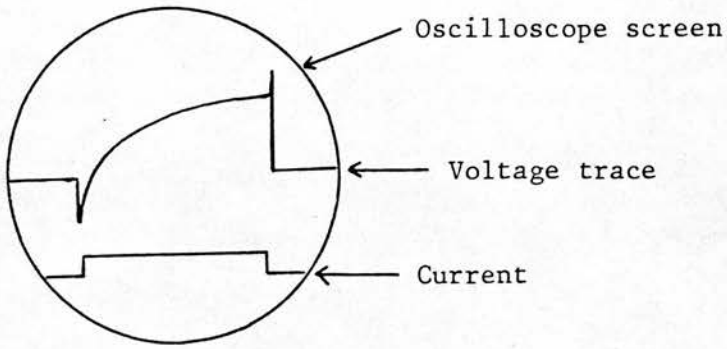
10) The oscilloscope voltage channel input was switched back to DC and the ME main box amplifier switched on. At this time it was normal to observe a ME tip potential of approximately 3 mV on the oscilloscope screen, and this was offset using the DC offset of the ME main box amplifier to bring the voltage trace back to zero on the oscilloscope screen. If the ME tip potential was greater than 3 mV, the ME was discarded.

11) 10 mV steps were applied using the DC calibrator to ensure that the apparatus was responding correctly.

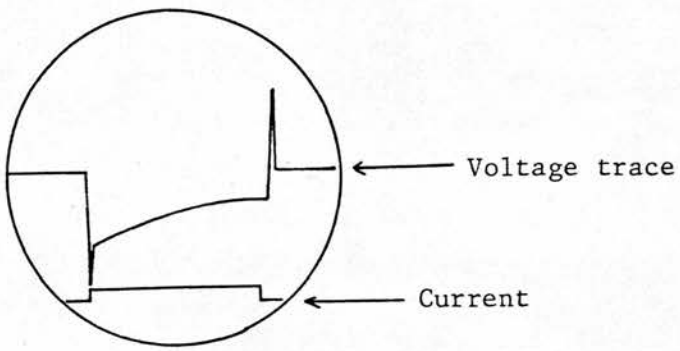
12) The stimulator output was adjusted in order to obtain a suitable current pulse through the ME of 100 msec duration and 2 nanoAmp amplitude The current pulse was monitored using the second beam of the oscilloscope.

13) At this time a deflection could be observed on the voltage trace, this was due to the Wheatstone Bridge being unbalanced because of the ME resistance (Figure 5.7a and b). The balance potentiometer of the main box was adjusted until the deflection was removed (Figure 5.7c) and the Wheatstone Bridge balanced. The value of the ME resistance could be read directly from the potentiometer balance; the ME was discarded if the resistance value was less than 20 MΩ or greater than 40 MΩ.

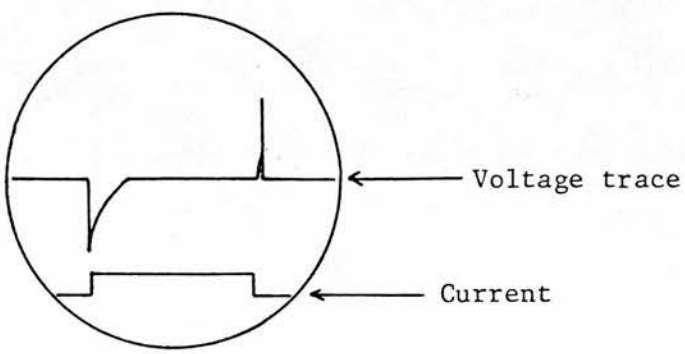
14) The ME, under visual and auditory control, was inserted into a cell using the micromanipulator. The MP was assessed by measuring the voltage change directly from the screen and by using the DC calibrator. It could be observed from the trace on the oscilloscope that insertion of the ME into the cell caused the Wheatstone Bridge to become unbalanced because of i) a resistance due to the ME clogging with protein and ii) the resistance of the cell membrane (Figure 5.8b). In order to correct for the increase in ME resistance, the difference between the ME and membrane time constants on the voltage pulse was used. On this pulse, the rise time due to the ME was shorter than that due to the cell membrane, thereby allowing the two components to be distinguished. The Wheatstone Bridge was balanced using the potentiometer on the main box, to allow for the extra resistance due to the ME (Figure 5.8c) and a reading R1 MΩ was noted. The Wheatstone Bridge was balanced again to allow for the resistance due to the cell membrane (Figure 5.8d) and a reading of R2 MΩ was noted. The cell IR was calculated by subtracting R1 from R2. The MP and IR values were recorded only if i) the recording remained stable for 60 secs on the oscilloscope screen and ii) the voltage beam returned to the zero base line upon



a) Voltage Trace Deflected Due to Resistance of ME Causing Imbalance of Wheatstone Bridge.

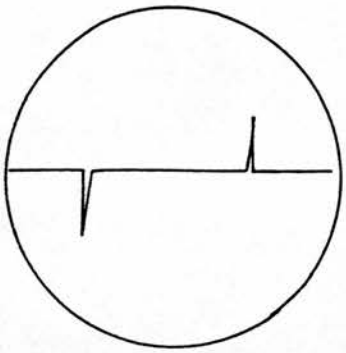


b) Voltage Trace Deflected Due to Resistance of ME Causing Imbalance of Wheatstone Bridge.



c) Voltage Trace Deflection Removed Due to Balancing of Wheatstone Bridge.

Figure 5.7

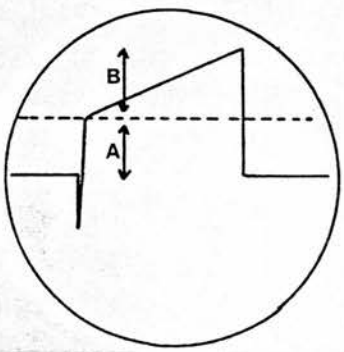


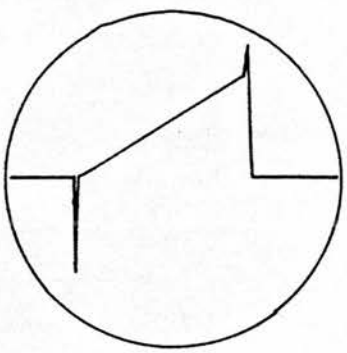
a) Oscilloscope Trace: Wheatstone Bridge Balanced Prior to Entry of ME Into Cell.

b) Oscilloscope Trace: Wheatstone Bridge Unbalanced Due to Resistance of Clogged ME Plus Cell IR.

A = resistance due to clogged ME

B = resistance due to cell membrane





c) Oscilloscope Trace: Wheatstone Bridge Balanced to Allow for Extra Resistance Due to ME.

Potentiometer Reading = R1 MΩ.

d) Oscilloscope Trace: Wheatstone Bridge Balanced to Allow for Resistance Due to Cell Membrane.

Potentiometer Reading = R2 MΩ.

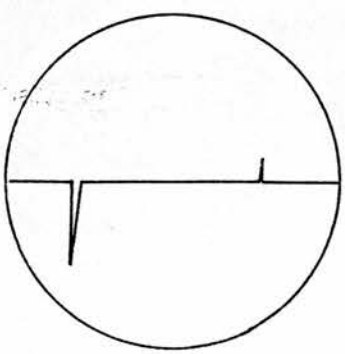


Figure 5.8.

removal of the electrode from the cell thereby indicating that the electrode had not become permanently blocked with protein.
15) The ME was removed from the cell and the Wheatstone Bridge re-balanced. If the resistance value for the ME had altered considerably from its original value due to either a broken tip or excessive clogging of the tip with protein the ME was discarded.
16) The above procedure was repeated using another cell. Only those cells that were completely isolated from each other were examined, and care was taken not to impale the same cell more than once. Ten cells per plate were examined for each treatment and time point. In general, it took 10 mins to assess one plate, in this time period it was found that any alteration in the pH value or temperature of the medium did not result in an alteration in the MP or IR (MO Wright, personal communication), therefore any observed changes in MP or IR could be assumed to be due to the treatment condition.
17) When 10 readings had been recorded from a plate, the viability of the cells was assessed using the Trypan Blue exclusion test (Section 4.2.3.4a). The results were expressed as the mean number of viable cells per 10 fields of view.

5.2.4 Statistical Analysis of Data.

The 10 readings from each culture dish were presented as a mean value ± SD. The Student T test was employed to assess the statistical significance of any differences between results (Bailey, 1974).

5.2.5 The Culture of P388D$_1$ Cells and Assessment of Their Suitability for Experimental Use.

The P388D$_1$ cells were harvested as described previously (Section 4.2.3.1), and plated in 50 mm tissue culture dishes at a concentration of 2.5×10^5 cells per dish. The cells were cultured in F10 with 12.5 mM morpholinopropanesulfonic acid (MOPS) (Eagle, 1971; W Christie, personal communication) and 4% heat inactivated newborn calf serum (HI NCS). The use of MOPS ensured that the pH value of the medium altered only 0.03 pH units during the electrophysiological examination of each plate (Gormley et al, 1978). The dishes were incubated for 24 hrs at 37 °C in a humidified atmosphere of 5% CO_2 in air, in order

to ensure that the cells had adhered firmly to and spread onto the plastic. The MP and IR values for 10 cells from an initial control plate were examined in order to establish their suitability for experimental use. In general, MP values of -10 to -13 mV and IR values of 10 to 40 MΩ were considered acceptable.

5.2.6 Preparation of Conditioned Medium for Use in Experiments Requiring the Use of Serum.

For some experiments, it was necessary to use medium containing serum in order to examine the role of serum in protecting $P388D_1$ cells against the cytotoxic action of fibres. The addition of fresh serum to cells can cause a cell membrane depolarisation (Bard and Wright, 1974; Hulser and Frank, 1971; Villereal, 1982); to overcome this effect, any dusts added to $P388D_1$ cells were suspended in conditioned medium (Wright and Gormley, 1980). This medium was prepared by incubating 4% HI NCS in F10 and MOPS in the presence of $P388D_1$ cells which were 70% confluent. After 24 hrs the conditioned medium was centrifuged at 500 g for 10 mins to removed cellular debris, and the resulting supernatant used for addition to the cells.

5.2.7 The Preparation of Fibrous Samples, Their Addition to $P388D_1$ Cells and Assessment of Their Effect on MP and IR.

All of the fibrous dust samples examined were prepared as described in Section 4.2.3.4. The concentration of the dusts was adjusted to either 10 or 50 μg/ml in F10 + MOPS alone, or 50 μg/ml in conditioned medium. Serum-free medium was used so that the direct effect of fibres on cells could be examined, and conditioned medium was utilised to examine the protective role of serum.

Following examination of the initial control, the plates were washed with F10 and treated with the appropriate medium containing fibres (test plates) or medium alone (control plates). The dishes were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. The plates were tested at hourly intervals after the addition of dust, for up to 6 hrs. Plates treated with dust and serum-free medium were examined for up to 6 hrs only; plates treated with dust and conditioned

medium were examined over a period of 3 days.

Samples of UICC crocidolite, amosite and chrysotile were examined at a concentration of 50 μg/ml in serum-free medium and also conditioned medium. Following the observation that UICC amosite could elicit a membrane hyperpolarisation in either the presence or absence of serum, a number of amosite samples from different sources were also studied to establish their abilities to elicit a membrane hyperpolarisation. The samples included E UICC amosite, E F amosite, E LF amosite and SF amosite at a concentration of 50 μg/ml of serum-free medium. UICC amosite was examined at a concentration of 10 μg/ml of serum-free medium to see if the hyperpolarisation occurred at a concentration lower than 50 μg/ml of serum-free medium.

5.2.8 The Ability of Leached UICC Amosite to Elicit a Cell Membrane Hyperpolarisation.

In order to establish whether the "hyperpolarising agent" was a contaminant material or an integral part of the structure of the amosite fibre, amosite fibres were leached in medium for various periods of time and the effect of both the leached fibres and the leachate on cell membrane electrophysiology was established.

10 mg samples of UICC amosite were suspended in 10 mls of F10 + MOPS, ultrasonicated for 2 mins as described previously (Section 4.2.3.4) and maintained in Universal tubes at 37 °C for periods of 4, 7, 25 and 30 days. After the appropriate period of time each tube was centrifuged at 800 g for 15 mins to sediment the fibres, although it must be borne in mind that the very small and fine fibres may have remained suspended. The supernatant leachate was harvested; the leached fibres were resuspended in fresh F10 + MOPS at a concentration of 50 μg/ml. The effect of the leachate and suspended fibres in the absence of serum on $P388D_1$ cell MP and IR was examined 1 hr after treatment, and compared with the effect of treatment with control medium or unleached UICC amosite.

5.2.9. The Ability of Manganese Chloride to Elicit a Cell Membrane Hperpolarisation.

Morgan and Cralley (1973) demonstrated that Mn^{++} contaminates UICC amosite at a level of 13000 ppm. In order to establish if Mn^{++} could be responsible for the induction of the cell membrane hyperpolarisation, the effect of adding manganese chloride ($MnCl_2$) to $P388D_1$ cells was examined. $MnCl_2.4H_2O$ was dissolved in F10 + MOPS (serum-free) and added to $P388D_1$ cells to give a final concentration of 12.6, 5.8, 2.9 or 0.7 µg per 5 mls per plate (12.6 µg/plate was the equivalent amount of Mn^{++} that would be found on UICC amosite fibres added at a concentration of 50 µg/ml). After incubation for 1 hr at 37 °C, the cell MP and IR values were recorded and compared with the Mn^{++}-free medium control. In order to establish if serum had a protective effect, $MnCl_2.4H_2O$ was added at a concentration of 12.6 µg/5 mls of conditioned medium to $P388D_1$ cells and the culture plate was incubated at 37 °C. Any changes in MP and IR were assessed at 20 minute intervals for up to 4 hrs and compared with the Mn^{++}-free medium control.

5.2.10 The Role of the Calcium-Sensitive Potassium Channel in the Induction of the Cell Membrane Hyperpolarisation.

A number of experiments were carried out to establish if the amosite and Mn^{++} induced cell membrane hyperpolarisation were due to the activation of the Ca^{++} sensitive K^+ conductance channel. It was necessary, however to demonstrate that $P388D_1$ cell membrane contained this channel. Acebutalol (May and Baker) which is known to block β-adrenergic receptors (Basil et al, 1973, 1974) and activate the Ca^{++} sensitive K^+ channel (MO Wright, personal communication), was added at a concentration of 40 µg/ml of serum-free medium to $P388D_1$ cells. After incubation at 37 °C for 10 mins the MP and IR values for the treated cells was examined. Quinidine-HCl (Sigma), which is known for its ability to block the Ca^{++} sensitive K^+ channel (Lew and Ferreira, 1979) was added to $P388D_1$ cells in F10 + MOPS (20 µM solution). After incubation at 37 °C for 10 mins the MP and IR values for the treated cells were examined. In order to establish the ability of quinidine to inhibit

the acebutalol-induced membrane hyperpolarisation, the $P388D_1$ cells were exposed to quinidine (20 μM solution) for 10 mins, followed by acebutalol (40 μg/ml) in serum-free medium. After 10 mins the cellular MP and IR values were examined

In order to establish the ability of quinidine to inhibit the Mn^{++} and amosite induced hyperpolarisation, the $P388D_1$ cells were exposed to quinidine (20 μM) for 10 mins at 37 °C, followed by treatment with $MnCl_2.4H_2O$ (12.6 μg/plate) for 30 mins at 37 °C or UICC amosite (50 μg/ml) for 60 mins at 37 °C. The cell MP and IR values were established for each treatment condition after the appropriate period of time, and compared with the results for the quinidine-free plates treated in parallel with Mn^{++} or UICC amosite.

5.3 RESULTS.

5.3.1 The Resting Potential and Input Resistance of the $P388D_1$ Cell in the Presence and Absence of Serum.

In general, the mean MP for the "initial control" cells varied between -6.5 and -13.5 mV and the mean IR value varied between 5 and 34 MΩ; the mean values never remained constant between experiments and some inter-experimental variation was always observed.

In general, exposure of the $P388D_1$ cells to the medium without serum, did not result in a significant alteration in either the MP or IR (see control values in Figures 5.13 to 5.15 and 5.19 to 5.23). However, on occasions an increase in both MP and IR was observed (Figures 5.9 and 5.10) or alternatively a decrease in both parameters occurred; this highlighted the requirement that control plates of $P388D_1$ cells should be examined in parallel with the treated plates to ensure that any observed alteration in cell membrane electrophysiological properties were due to the treatment alone.

For those experiments requiring serum, treatment of $P388D_1$ cells with conditioned medium proved satisfactory. For up to 7 hrs following treatment (Figure 5.11 and 5.12) no significant alteration in either MP or IR was observed;after the 50 hrs time point onwards however a considerable perturbation of both MP and IR was sometimes noted which was presumed to be due to the exhaustion of the nutrient components of the medium.

5.3.2 The Effect of UICC Asbestos Samples, in the Presence and Absence of Serum, on the $P388D_1$ Cell Membrane Electrophysiological Properties.

The MP and IR values for $P388D_1$ cells following treatment with UICC crocidolite, amosite or chrysotile at a concentration of 50 μg/ml, in the absence of serum, are shown in Figures 5.13 to 5.15. UICC crocidolite had little effect on either MP or IR during the 6 hrs following treatment (Figure 5.13a and b), and this was consistent with the high viability of the treated cells (Figure 5.13c). UICC amosite

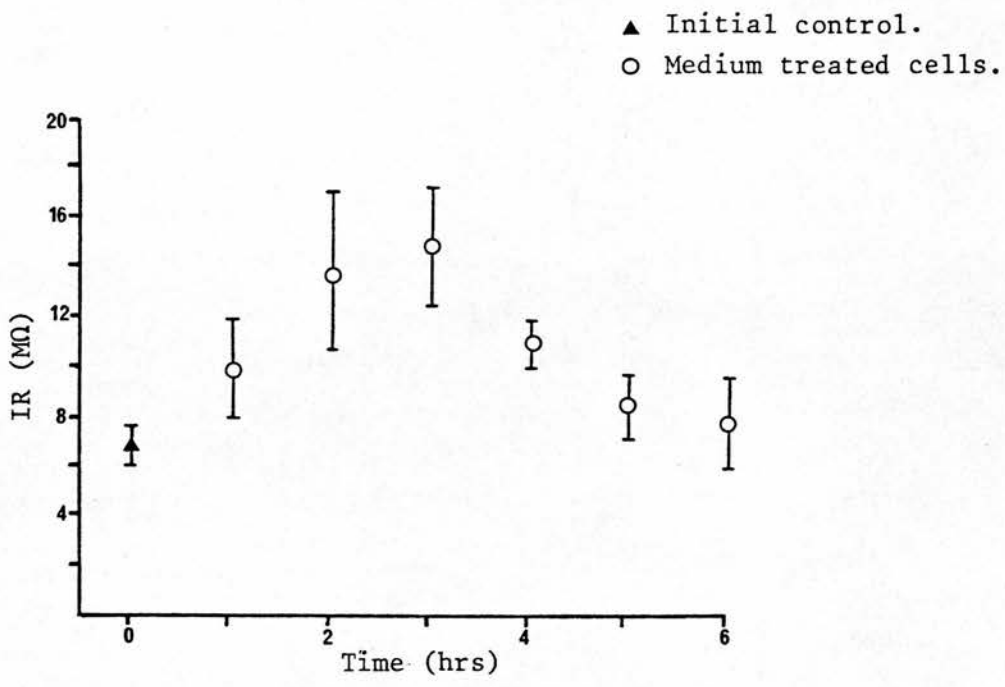


Figure 5.9 The Resting Potential of P388D$_1$ Cells During Treatment With Serum-Free Medium.

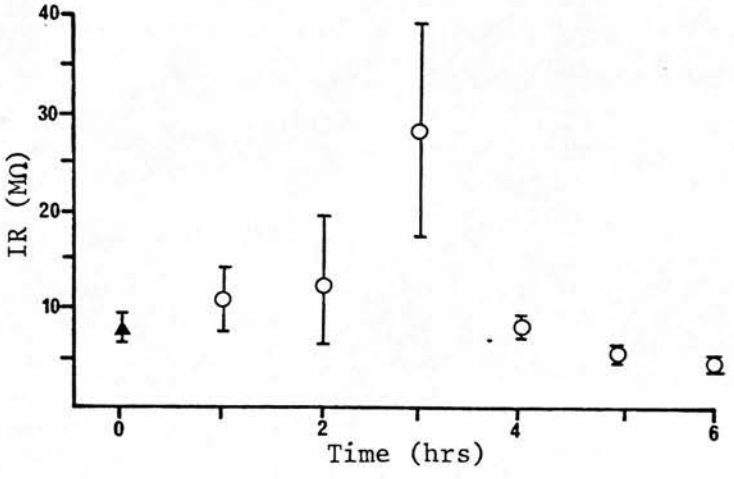


Figure 5.10 The Input Resistance of P388D$_1$ Cells During Treatment With Serum-Free Medium.

Each value is a mean of readings obtained from 10 cells ± SD.

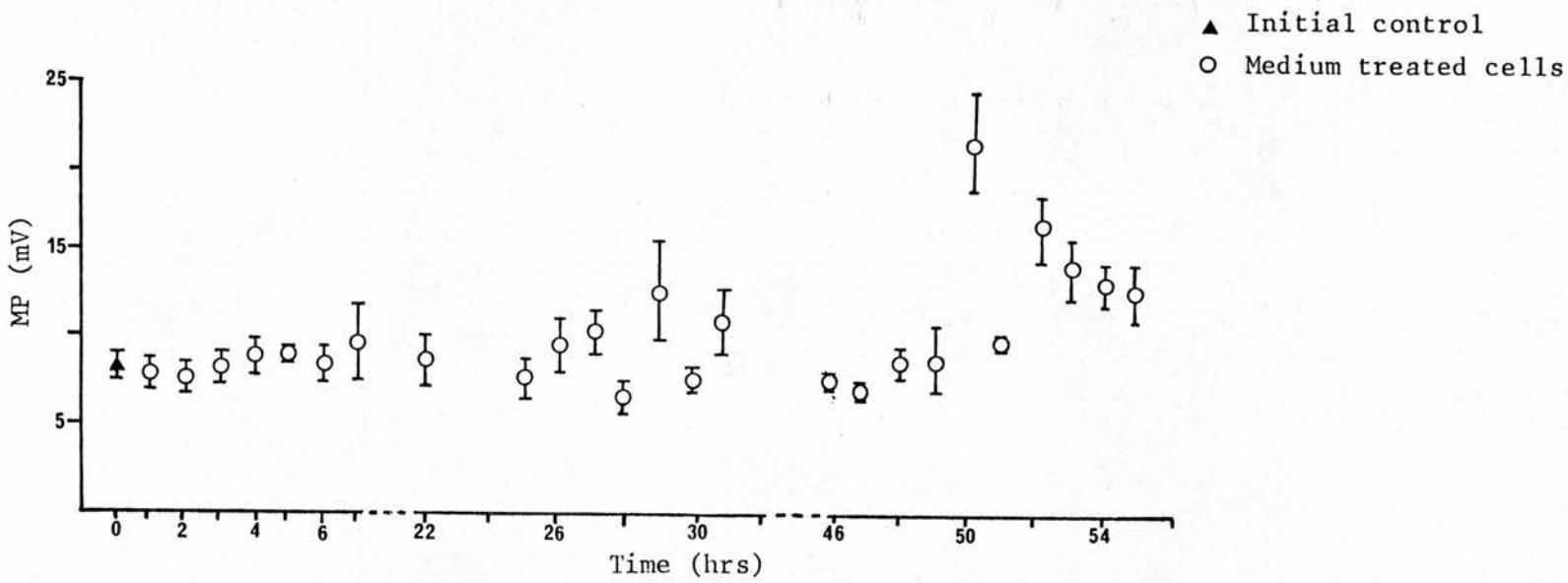


Figure 5.11 The Resting Potential of P388D$_1$ Cells During Treatment With Conditioned Medium.

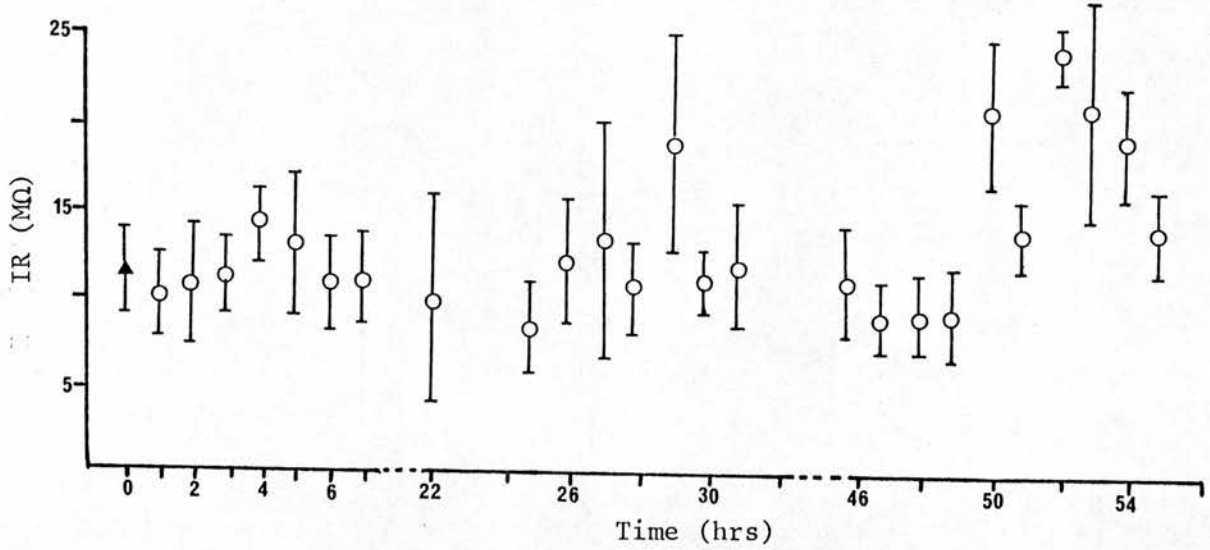
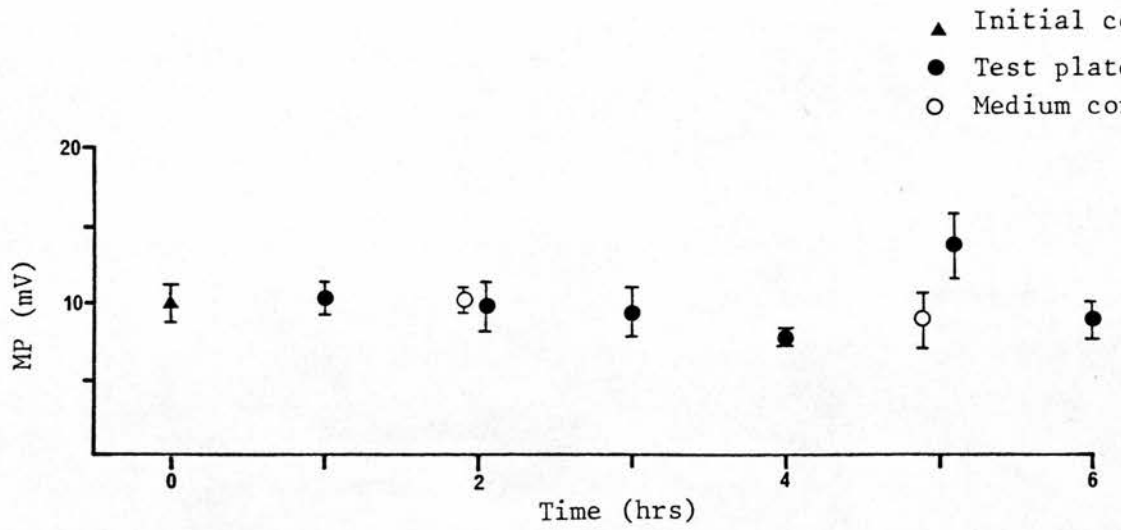
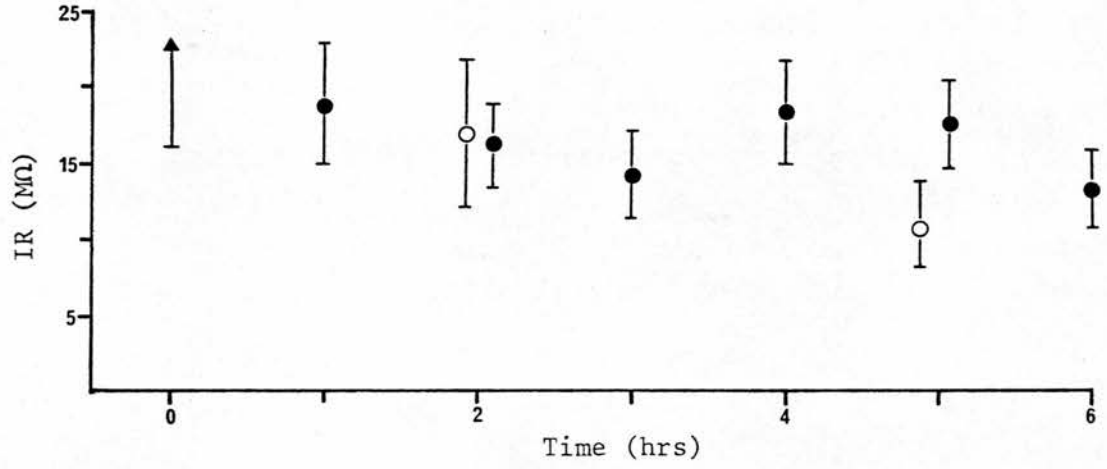


Figure 5.12 The Input Resistance of P388D$_1$ Cells During Treatment With Conditioned Medium.

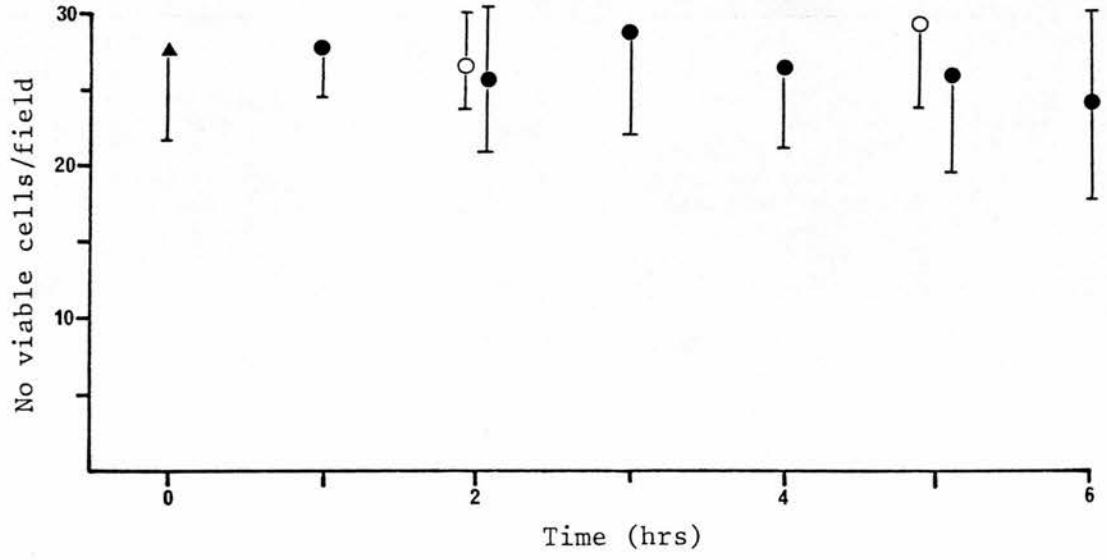
Each value is a mean of 10 readings ± SD.



a) Membrane Potential.



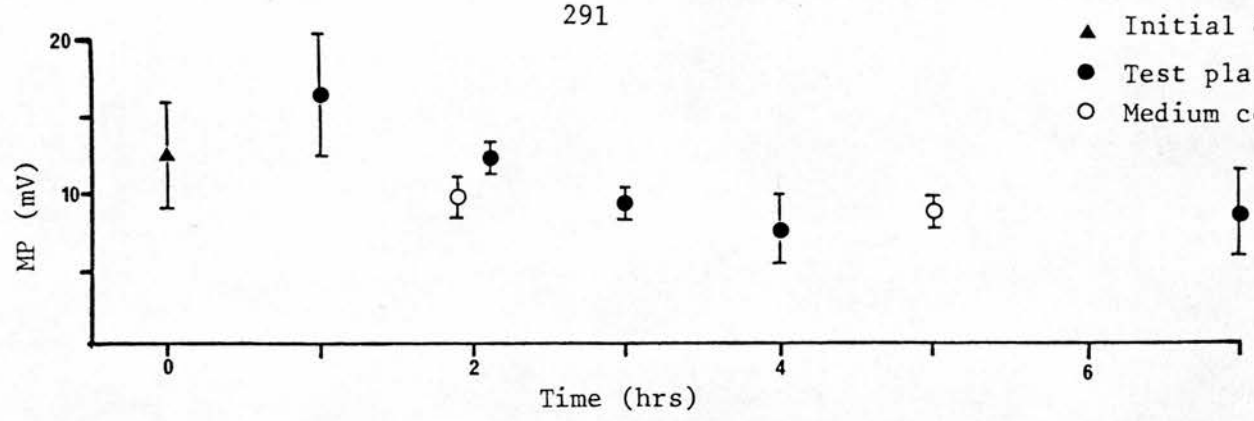
b) Input Resistance.



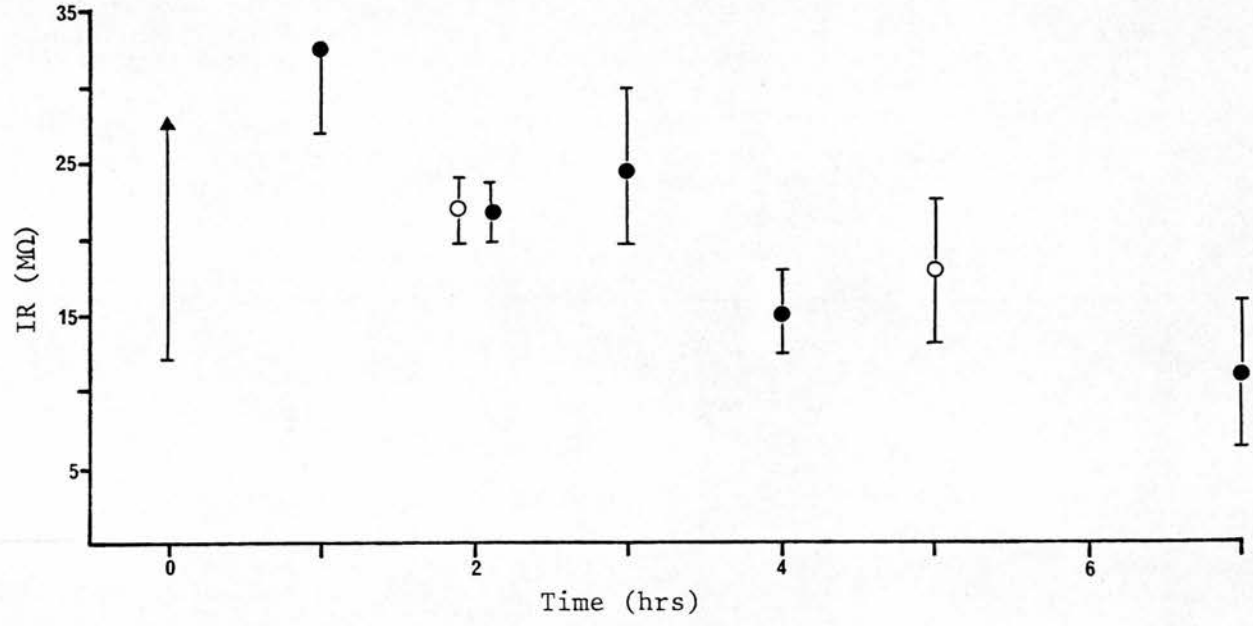
c) Number of Viable Cells/Field.

Figure 5.13 The Response of P388D$_1$ Cells During Treatment With UICC Crocidolite (50 µg/ml) in the Absence of Serum.

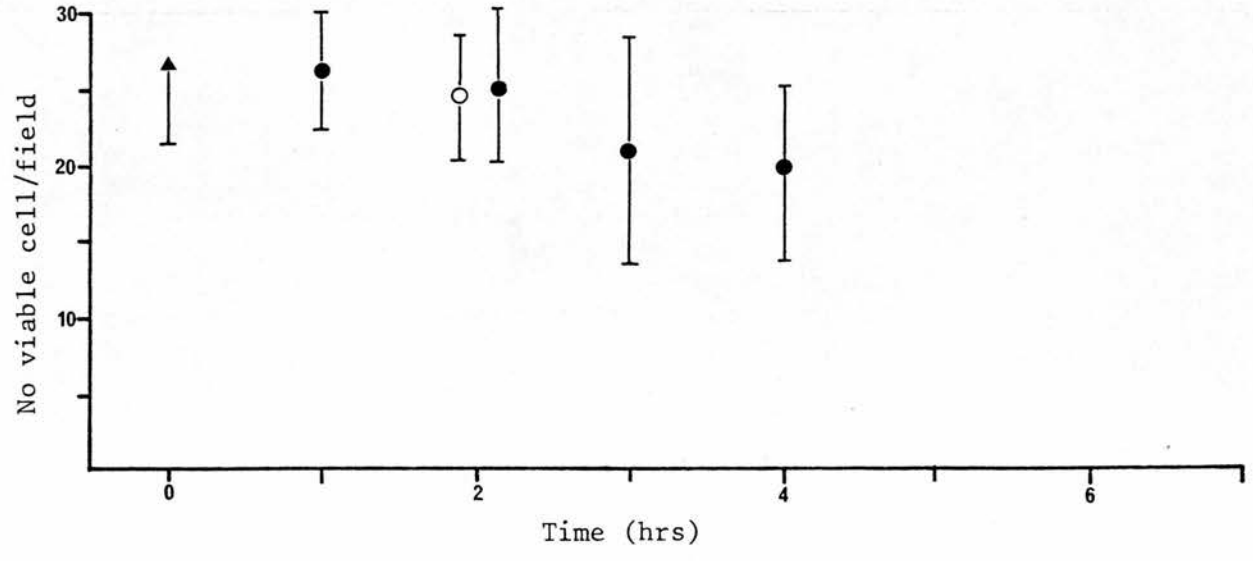
Each value is a mean of 10 reading ± SD.



a) Membrane Potential.



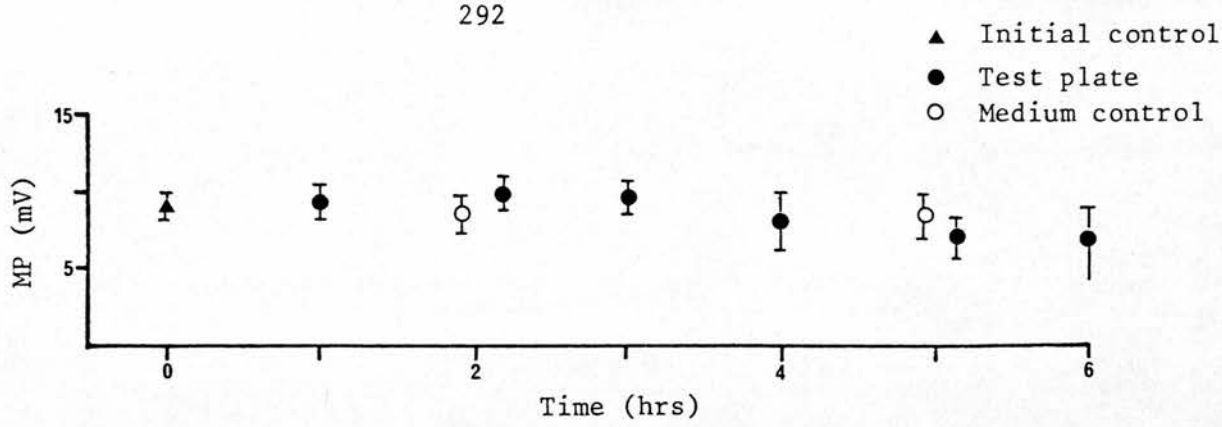
b) Input Resistance.



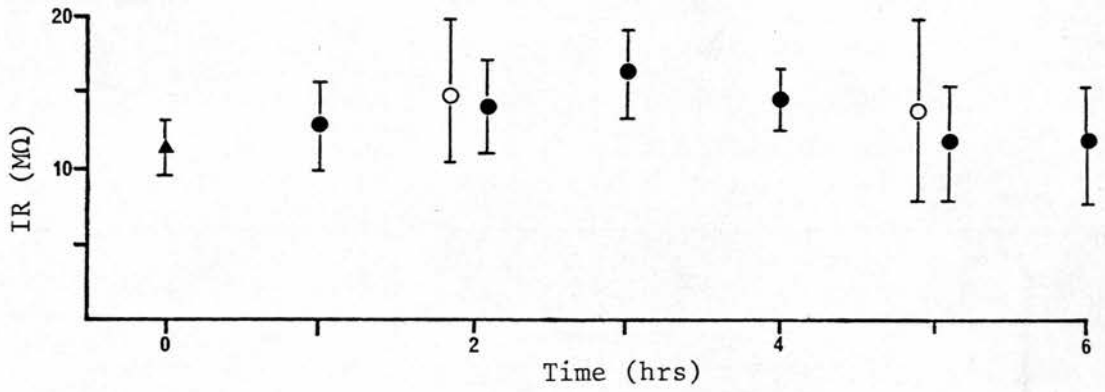
c) Number of viable cells/field.

Figure 5.14 Response of P388D$_1$ Cells During Treatment With UICC Amosite (50 μg/ml) in the Absence of Serum.

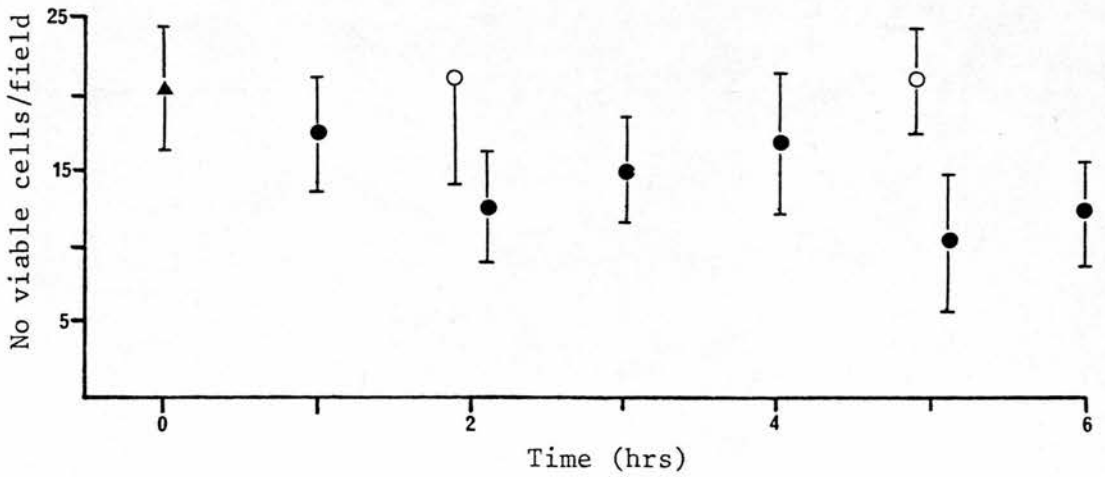
Each value is a mean of 10 readings ± SD.



a) Membrane Potential.



b) Input Resistance.



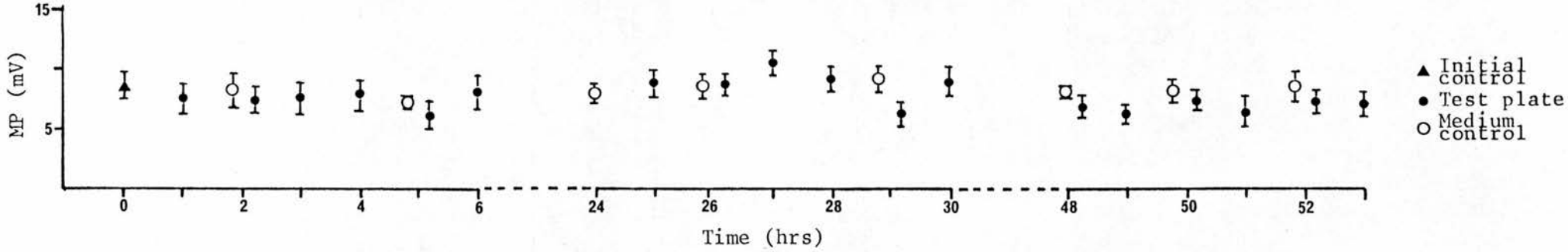
c) Number of Viable Cells/Field.

Figure 5.15 Response of P388D$_1$ Cells During Treatment With UICC Chrysotile (50 μg/ml) in the Absence of Serum.

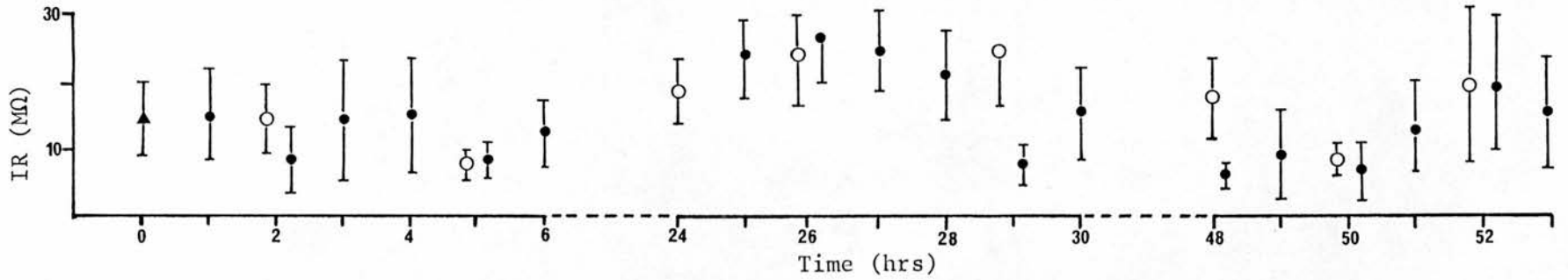
Each value is a mean of 10 readings ± SD.

however, (Figure 5.14) showed the ability to initiate a cell membrane hyperpolarisation 1 hr after addition to the $P388D_1$ cells, and a significant increase in both MP and IR was seen ($p<0.001$ between both MP and IR values for UICC amosite treated and medium control at 1 hr time point); the hyperpolarisation reached a peak value at the 1 hr time point (Figure 5.14a and b) and thereafter declined until the MP and IR values were similar to those obtained for the medium control cells. 7 hrs following treatment with UICC chrysotile the IR value fell below the control level($p<0.01$), although the MP remained stable, thereby suggesting an increase in the cell membrane permeability. Treatment of $P388D_1$ cells with UICC chrysotile had little effect on the cell membrane electrophysiology (Figure 5.15a and b); a significant decline in cell viability was observed at the 5 hrs time point (Figure 5.15c) ($p<0.001$ between UICC chrysotile treated and medium control cells), and this was reflected in a slight, but not significant, decrease in both MP and IR.

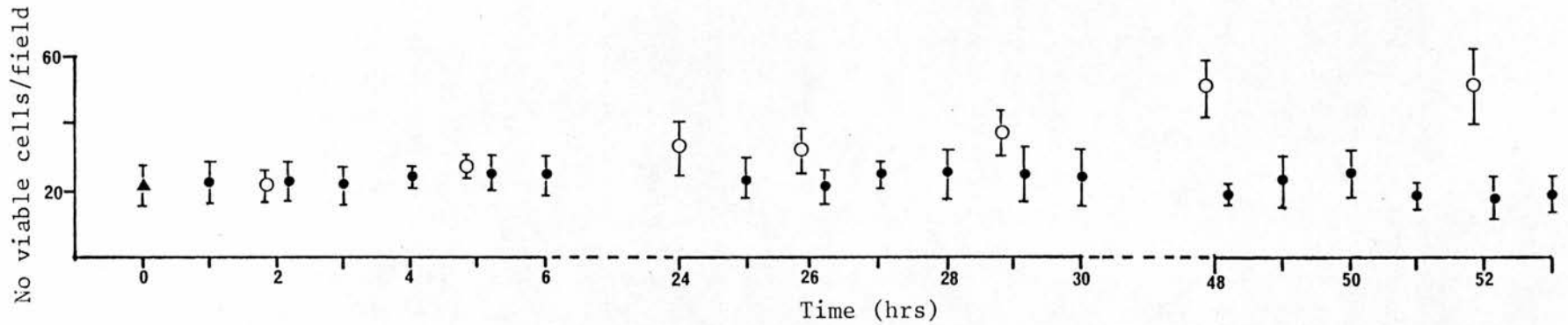
The $P388D_1$ cells were also exposed to UICC crocidolite, amosite and chrysotile (50 μg/ml) in the presence of conditioned medium containing 4% serum. The electrophysiological properties of the membranes of these treated cells were examined for up to 3 days following initial treatment, and the results are shown in Figure 5.16 to 5.18. Treatment with UICC crocidolite did not cause the values for either MP or IR to alter significantly from the values obtained for the medium controls (Figure 5.16a and b); a significant reduction in the number of viable cells was observed 48 hrs following exposure ($p<0.001$ between UICC crocidolite treated and medium control cells at 48 hrs), although the number of viable dust-treated cells per field was still similar to the number of cells per field noted for the initial control ie approximately 20 cells/field of view. The treatment of $P388D_1$ cells with UICC amosite (Figure 5.17) resulted in a significant increase in the MP at the 1 hr time point ($p<0.001$), although no alteration in IR was observed (Figure 5.17a and b). At the 2 hr time point the MP had declined to a level similar to that observed for the medium control cells. By 48 hrs, however, both MP and IR values were significantly lower than the control level ($p<0.001$ between both MP or IR values for UICC amosite treated and medium control cells at 48 hrs). The cell number counts



a) Membrane Potential.

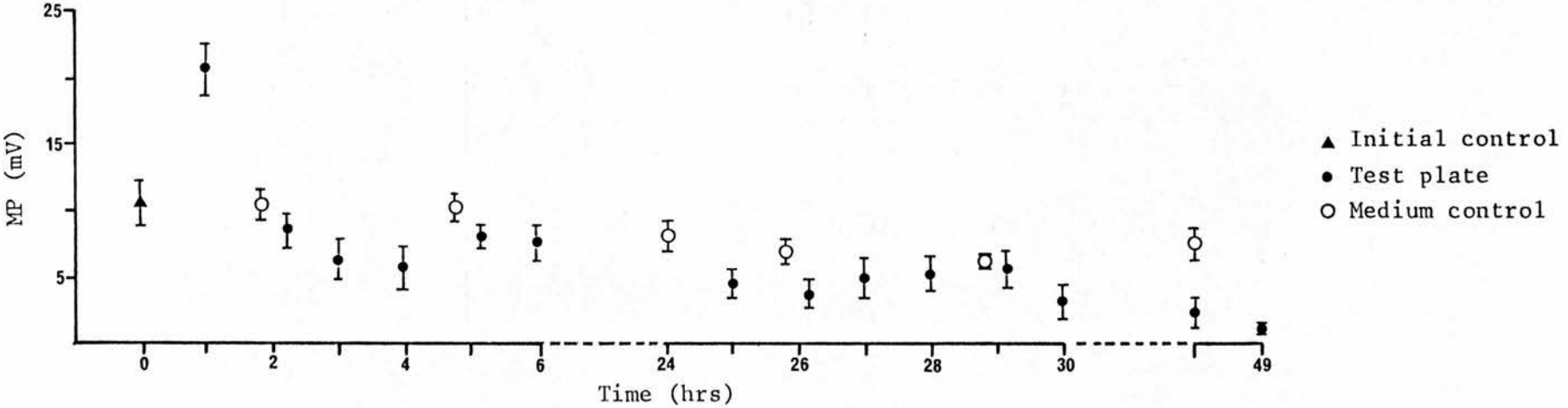


b) Input Resistance.

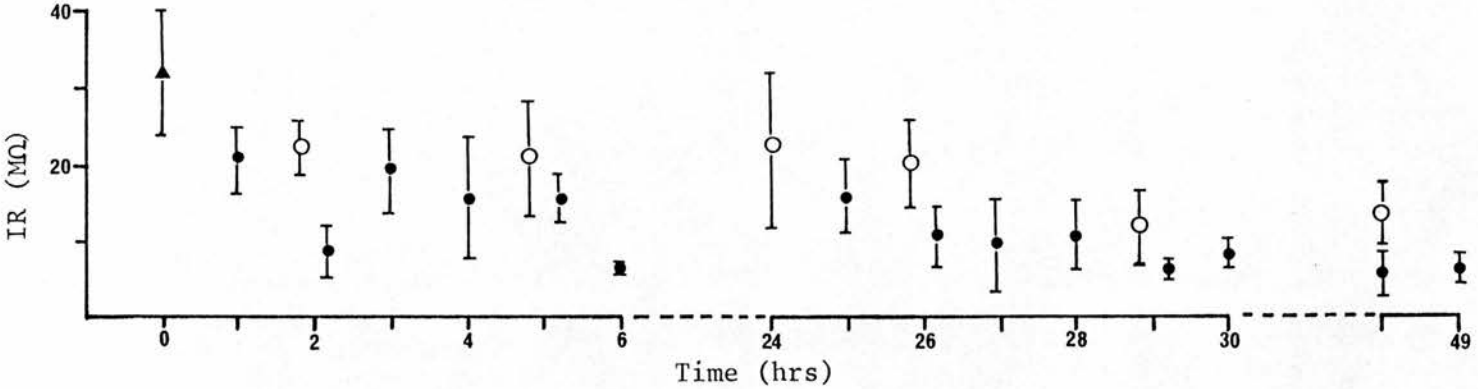


c) Number of Viable Cells/Field.

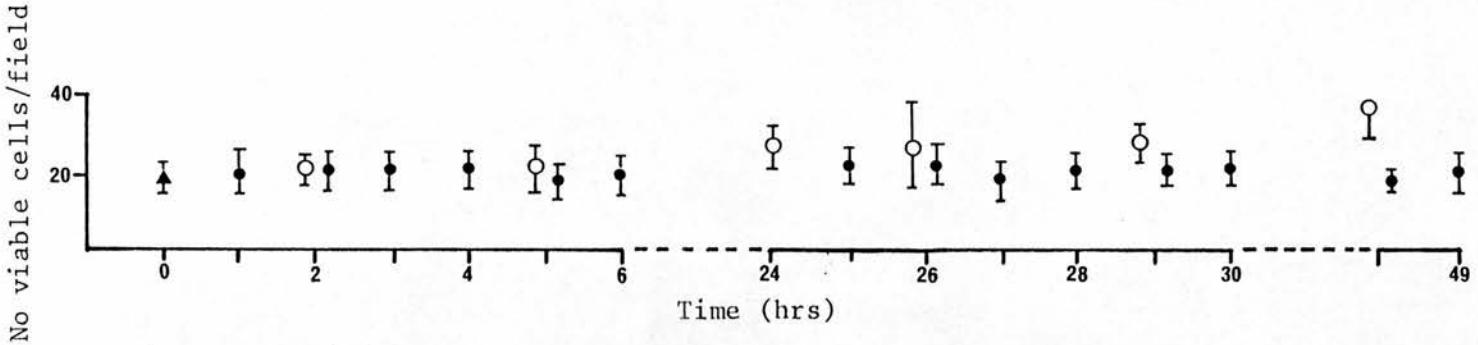
Figure 5.16 Response of P388D$_1$ Cells During Treatment With UICC Crocidolite (50 μg/ml) in the Presence of Serum.
Each value is a mean of 10 readings ± SD.



a) Membrane Potential.

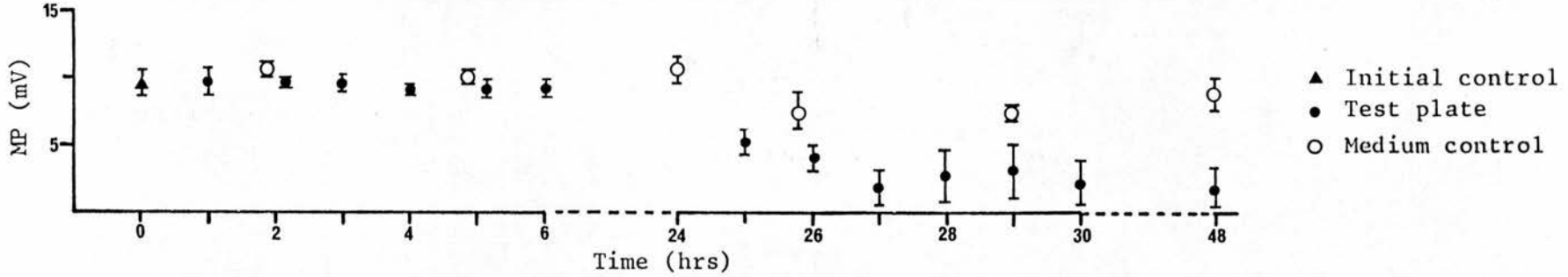


b) Input Resistance.

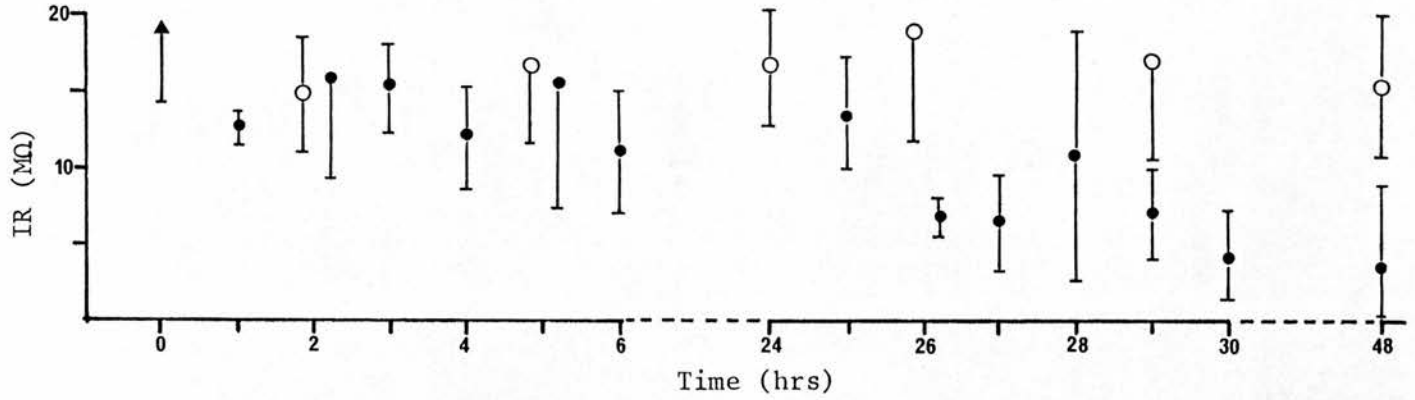


c) Number of Viable Cells/Field.

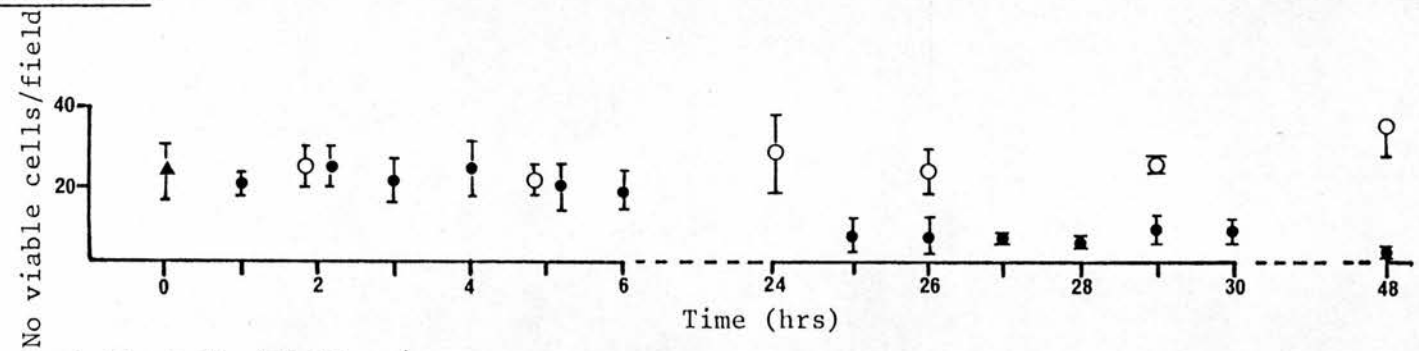
Figure 5.17 Response of P388D$_1$ Cells During Treatment With UICC Amosite (50 µg/ml) in the Presence of Serum.
Each value is a mean of 10 readings ± SD



a) Membrane Potential.



b) Input Resistance.



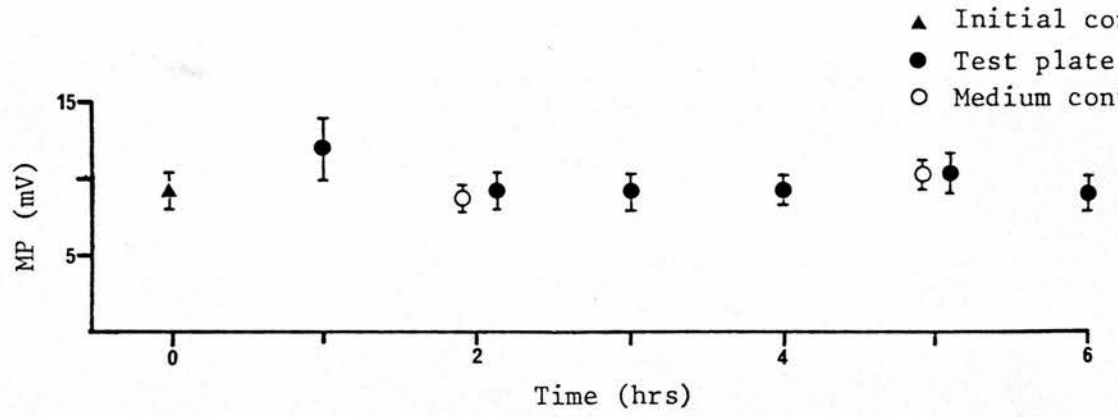
c) Number of Viable Cells/Field.

Figure 5.18 Response of P388D$_1$ Cells During Treatment With UICC Chrysotile (50 μg/ml) in the Presence of Serum.
Each value is a mean of 10 readings ± SD.

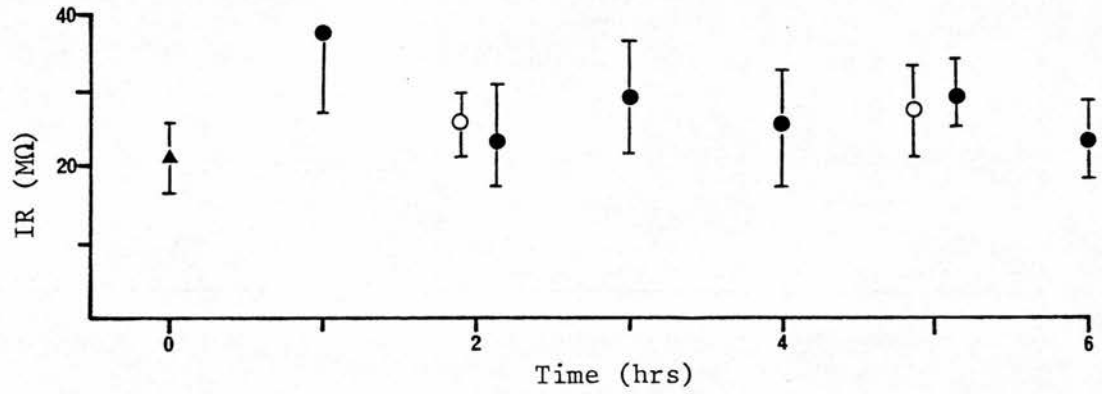
for the UICC amosite treated cells, whilst lower than the medium control counts (Figure 5.17c) ($p<0.001$) were still similar to values obtained for the initial control cells, thereby suggesting that the cells were not dead but had not divided. The treatment of the $P388D_1$ cells with UICC chrysotile fibres (Figure 5.18) resulted in a significant decline in both MP and IR values 26 hrs following exposure ($p<0.001$ between both MP and IR values for UICC chrysotile treated and medium control cells at 26 hrs), and this finding was coincident with a significant decline in the number of viable cells (Figure 5.18c) ($p<0.001$ between UICC chrysotile treated cells at 26 hrs and initial control cells). 48 hrs following treatment with UICC chrysotile, the values obtained for the MP, IR and viability parameters were low enough to show that extensive cell death had occurred.

5.3.3 An Investigation Into the Ability of Various Amosite Samples to Elicit a Cell Membrane Hyperpolarisation.

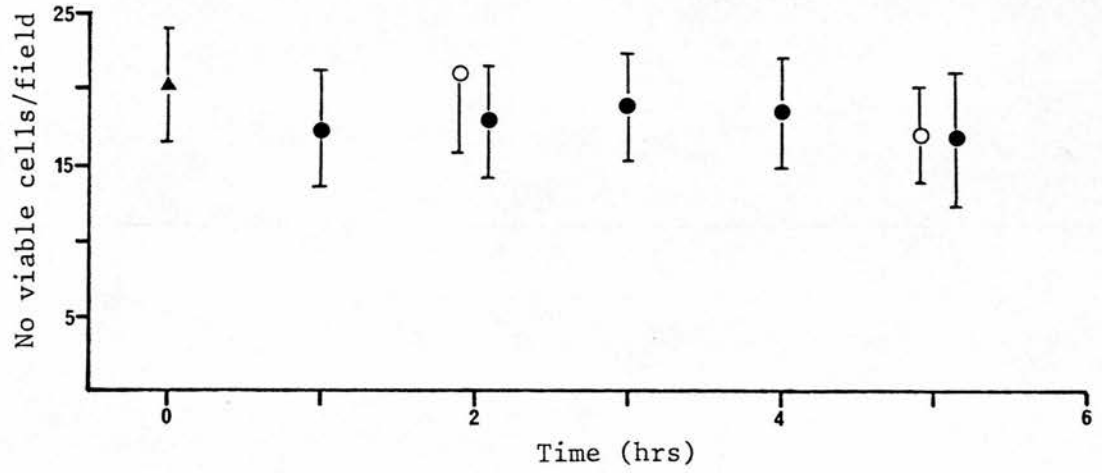
In order to discover whether concentration was important in the production of a cell membrane hyperpolarisation, $P388D_1$ cells were exposed to a lower concentration of UICC amosite (10 μg/ml) (Figure 5.19) in the absence of serum. 1 hr after exposure to the fibres a significant increase in both cell MP and IR values was noted ($p<0.01$ compared to control), thereafter the level decreased to the value obtained for the control cells and remained stable. The ability of amosite samples from different sources to elicit a cell membrane hyperpolarisation was also examined; $P388D_1$ cells were exposed to a dust concentration of 50 μg/ml in serum-free medium. E UICC amosite (Figure 5.20) induced a significant increase in both cell MP and IR ($p<0.02$ between both MP and IR values for E UICC amosite treated and medium control cells at 1 hr time point), this increase reached a maximum value 1 hr after exposure of the cells, and thereafter declined to the level observed for the control cells. The treatment of $P388D_1$ cells with E F amosite (Figure 5.21) did not result in the induction of a cell membrane hyperpolarisation until the 2 hr time point; a significant increase in the MP value was recorded ($p<0.001$ between E F amosite treated and control cells at 2 hr time point), the IR value had increased slightly but this did not differ significantly from the medium control.



a) Membrane Potential.



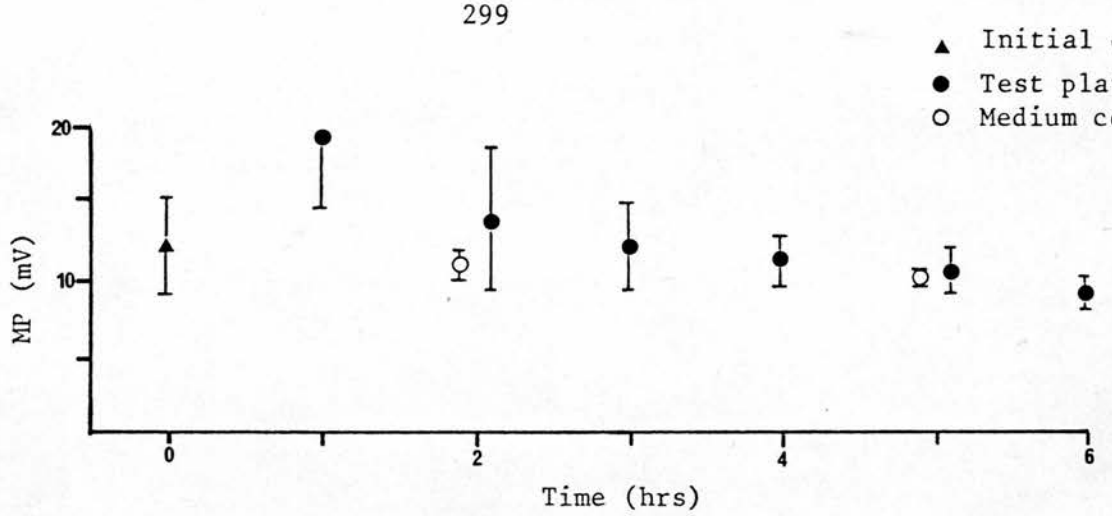
b) Input Resistance.



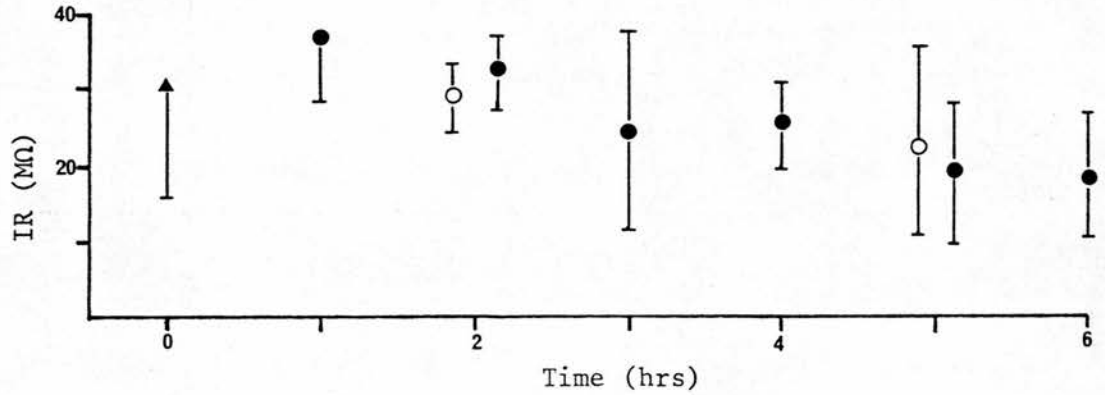
c) Number of Viable Cells/Field.

Figure 5.19 Response of $P388D_1$ Cells During Treatment With UICC Amosite (10 μg/ml) in the Absence of Serum.

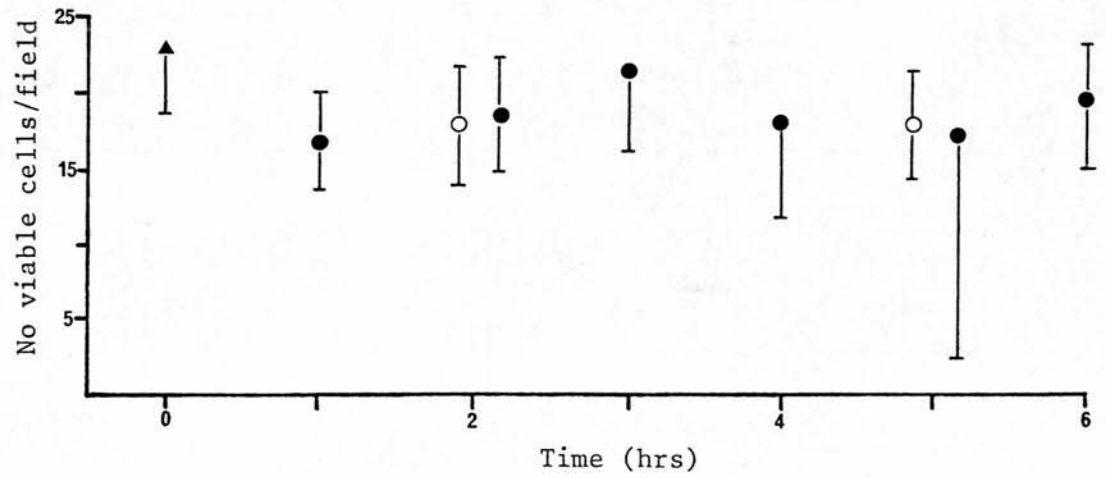
Each figure is a mean of 10 readings ± SD.



a) Membrane Potential.



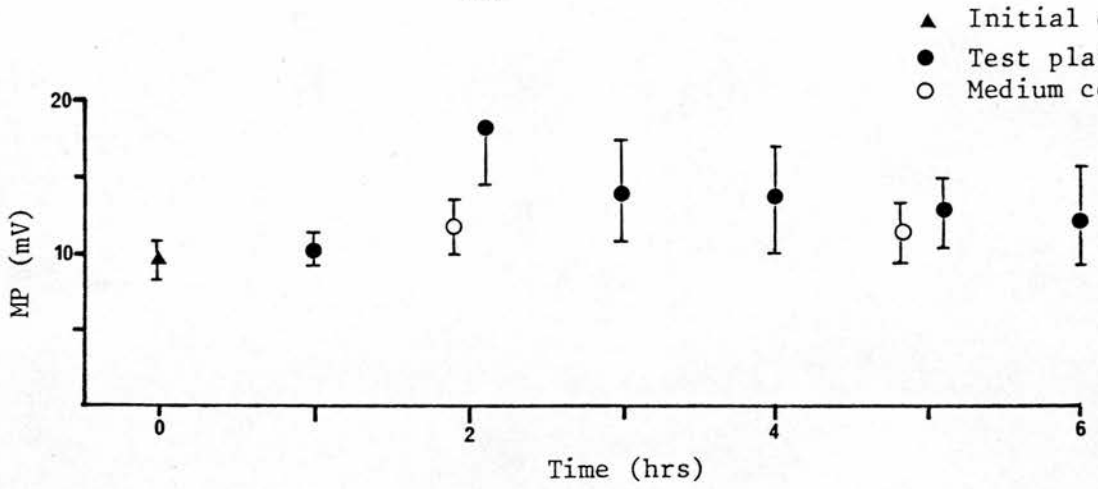
b) Input Resistance.



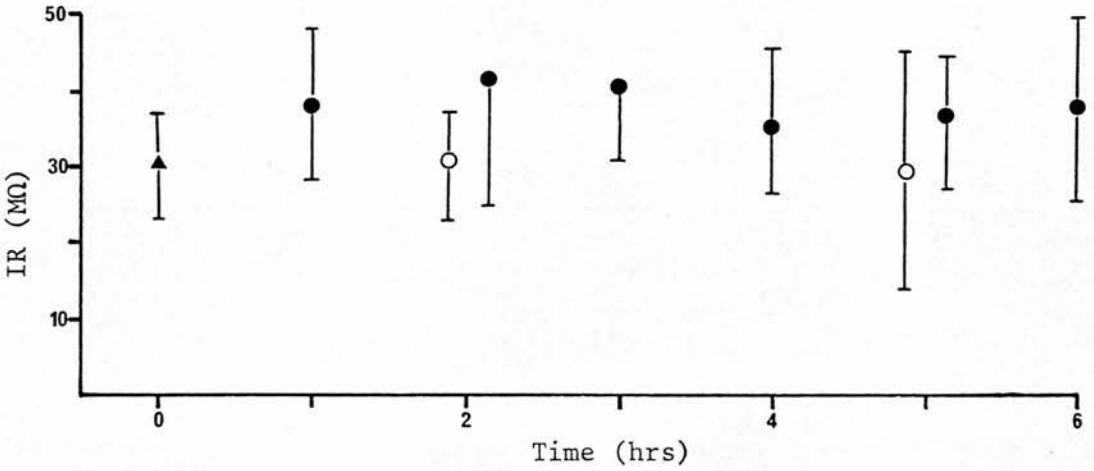
c) Number of Viable Cells/Field.

Figure 5.20 Response of P388D$_1$ Cells During Treatment With E UICC Amosite (50 μg/ml) in the Absence of Serum.

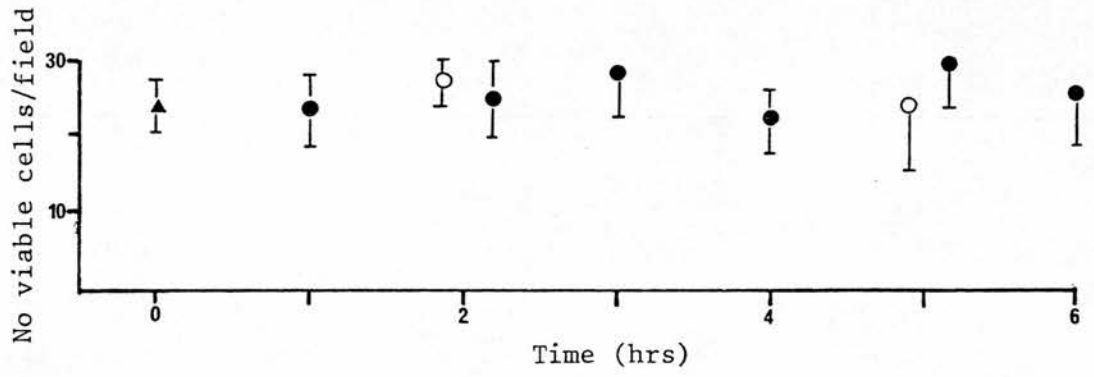
Each value is a mean of 10 readings ± SD.



a) Membrane Potential.



b) Input Resistance.



c) Number of Viable Cells/Field.

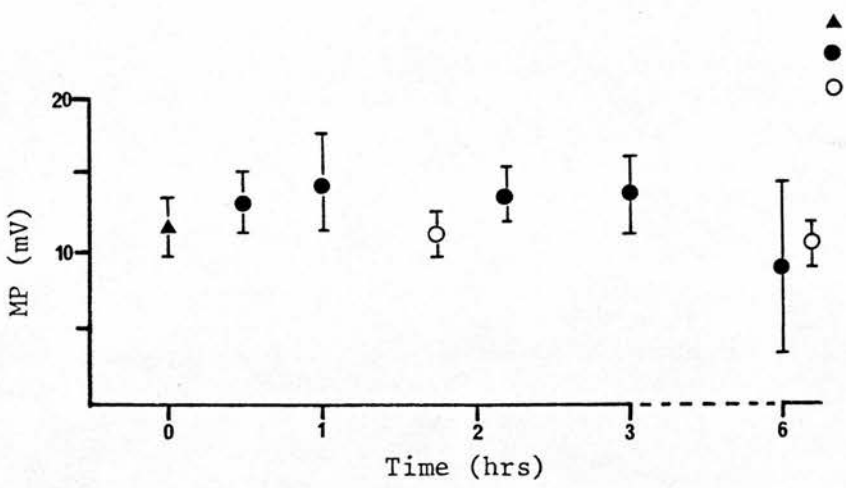
Figure 5.21 Response of P388D$_1$ Cells During Treatment With E F Amosite (50 μg/ml) in the Absence of Serum.

Each value is a mean of 10 readings ± SD.

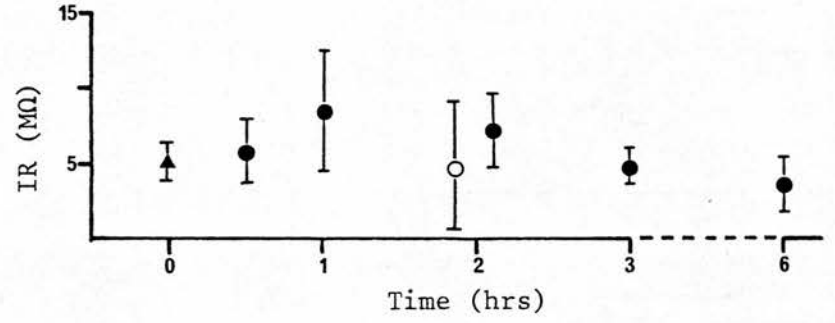
E LF amosite (Figure 5.22) also showed the ability to induce a $P388D_1$ cell membrane hyperpolarisation, causing a significant increase in both MP and IR after 1 hr of treatment ($p<0.05$ between both MP and IR values for E LF amosite treated and control cells at 1 hr time point). It should also be noted that 6 hrs following the exposure of $P388D_1$ cells to E LF amosite, the standard deviations around the mean value for the MP had increased considerably (Figure 5.22a), and this was consistent with the observed reduction in cell viability at this time point (Figure 5.22c) ($p<0.001$ between cell counts for E LF amosite treated and medium control cells at 6 hr time point). The SF amosite sample (Figure 5.23), however, had no noticeable effect on the $P388D_1$ cell MP, IR or viability count.

5.3.4 An Investigation Into the Ability of Leached Amosite Fibres and the Corresponding Leachate to Elicit a Cell Membrane Hyperpolarisation.

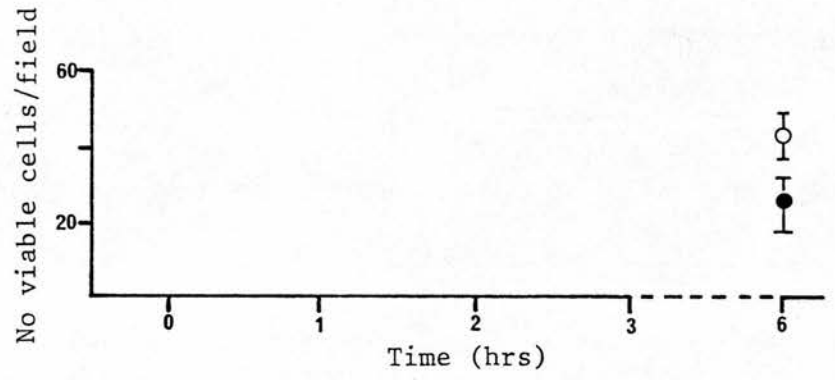
In order to establish if the agent responsible for eliciting the cell membrane hyperpolarisation was an integral part of the amosite fibre or a contaminating agent, UICC amosite fibres were leached in serum-free medium for various periods of time; $P388D_1$ cells were exposed to the leached fibres (50 μg/ml) or the corresponding leachate for 1 hr (Table 5.2). It was observed that UICC amosite fibres that had been leached for 4 days had the ability to induce a slight, but not significant, increase in the $P388D_1$ cell MP; after 7 days of leaching the fibres still retained the ability to elicit a slightly higher MP value than was observed for the medium control cells, although the standard deviations around the mean MP values for the 4 and 7 day leached samples were larger than those surrounding the mean values for the control cells. It was found that leaching the fibres for 25 days or longer effectively removed the ability of the samples to elicit any hyperpolarising activity, and the results obtained for the leached amosite samples were similar to those obtained for the medium control cells. The $P388D_1$ cells were also exposed to the supernatant leachate to establish if the "hyperpolarising agent" had leached into the fluid. The leachate obtained after 4 or 7 days of leaching showed the ability to increase the IR of the $P388D_1$ cell membrane ($p<0.02$ between IR values for leachate and medium control after 4 days of leaching;



a) Membrane Potential.



b) Input Resistance.

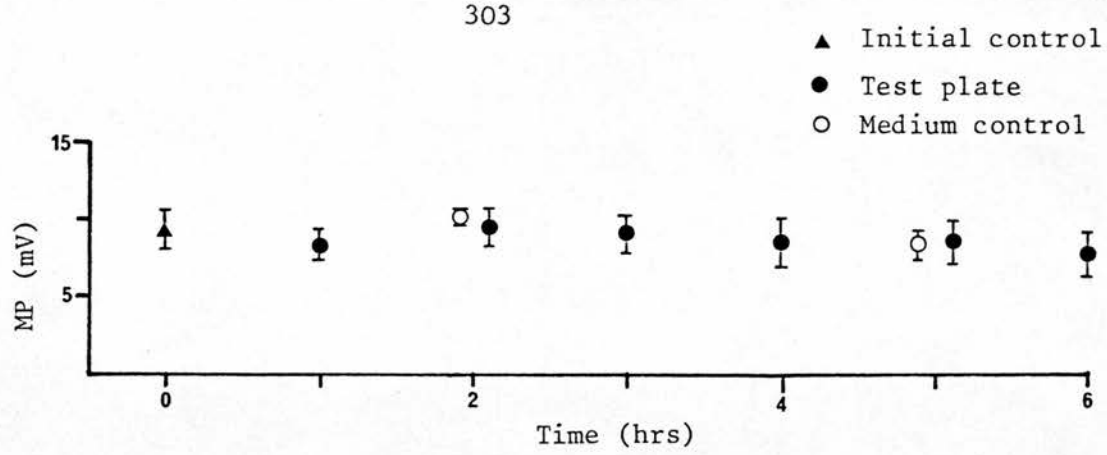


c) Number of Viable Cells/Field.

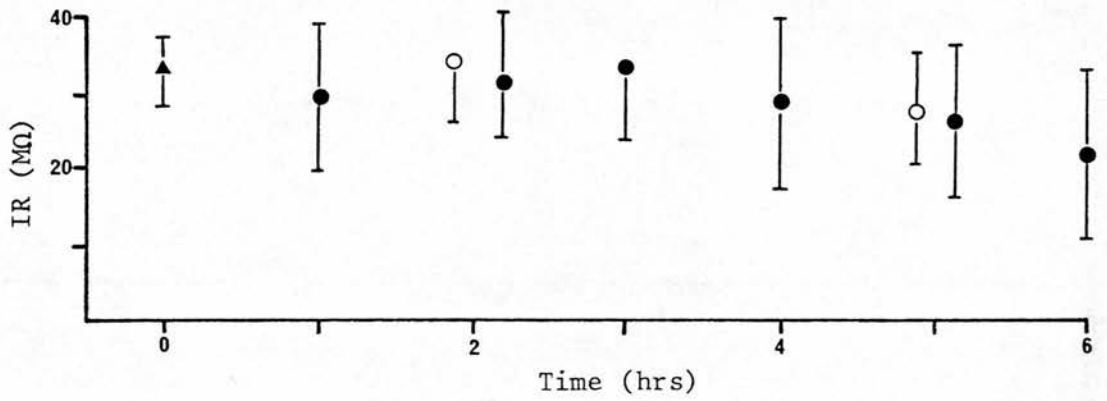
Figure 5.22 Response of P388D$_1$ Cells During Treatment With E LF Amosite (50 μg/ml) in the Absence of Serum.

Each value is a mean of 10 recordings ± SD.

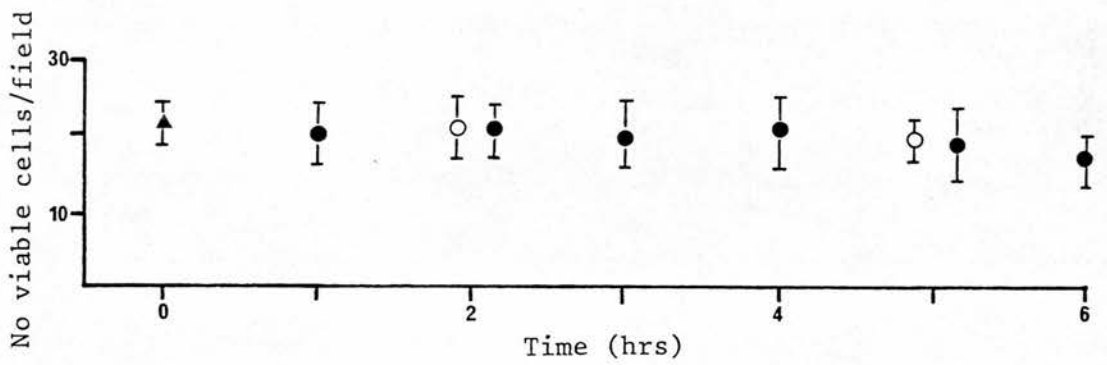
The complete data for part c) is not available due to experimental difficulties encountered in this study.



a) Membrane Potential.



b) Input Resistance.



c) Number of Viable Cells/Field.

Figure 5.23 Response of P388D$_1$ Cells During Treatment With SF Amosite (50 µg/ml) in the Absence of Serum.

Each value is a mean of 10 readings ± SD.

Table 5.2 The Response of $P388D_1$ Cells One Hour After Exposure to Either UICC Amosite, UICC Amosite Fibres Leached for Various Periods of Time, or the Corresponding Leachate in the Absence of Serum.

a) 4 Days Leaching.	MP (mV)	IR (MΩ)
Initial control	11.3 ± 3.2	7.3 ± 4.2
Leached amosite (50 μg/ml)	13.7 ± 4.0	6.6 ± 1.9
Leachate	11.8 ± 2.6	10.4 ± 5.1
Medium control	11.2 ± 1.0	5.2 ± 3.9
b) 7 Days Leaching.		
Initial control	12.2 ± 2.3	4.9 ± 0.7
UICC amosite (50 μg/ml)	17.3 ± 4.3	8.5 ± 5.7
Leached amosite (50 μg/ml)	14.9 ± 5.2	8.6 ± 7.2
Leachate	12.3 ± 2.8	12.7 ±12.4
Medium control	13.0 ± 3.7	4.6 ± 2.0
c) 25 Days Leaching.		
Initial control	12.7 ± 1.8	5.8 ± 1.9
UICC amosite (50 μg/ml)	16.0 ± 4.0	6.4 ± 3.8
Leached amosite (50 μg/ml)	12.6 ± 2.4	4.1 ± 1.5
Leachate	14.6 ± 2.4	4.6 ± 1.7
Medium control	13.8 ± 3.7	5.4 ± 3.4
d) 30 Days Leaching.		
Initial control	13.4 ± 1.8	5.5 ± 1.4
UICC amosite (50 μg/ml)	20.0 ± 6.0	8.8 ± 4.1
Leached amosite (50 μg/ml)	15.1 ± 2.7	5.4 ± 2.9
Leachate	20.4 ± 7.6	9.4 ± 6.3
Medium control	14.9 ± 2.9	5.9 ± 3.8

Each value is a mean of 10 readings ± SD.

although no significant difference was seen after 7 days of leaching); but the treatment did not result in an increase in MP. After 25 days of leaching, the supernatant leachate showed the ability to induce a slight, but not significant, increase in the $P388D_1$ cell MP; after 30 days, however, treatment of $P388D_1$ cells with the appropriate leachate resulted in a significant increase in MP ($p<0.05$ for 30 day leachate-treated and medium control cells) that was equivalent to the value obtained for the unleached UICC amosite induced cell membrane hyperpolarisation (Table 5.2d). This result demonstrated that the "hyperpolarising agent" can be removed from the amosite fibres by leaching and therefore may be a contaminating agent rather than an integral part of the structure of the fibre.

5.3.5 An Investigation Into the Ability of Manganese Ions to Induce a Cell Membrane Hyperpolarisation.

In order to test the hypothesis that the "hyperpolarising agent" may be a contaminating element, the effect of Mn^{++} on the $P388D_1$ cell membrane electrophysiology was studied, as Mn^{++} reputedly contaminates UICC amosite to a greater extent than the other UICC asbestos samples (Morgan and Cralley, 1973). The response of $P388D_1$ cells 1 hr following treatment with various concentrations of Mn^{++}, in the form of $MnCl_2.4H_2O$, in the absence of serum is shown in Table 5.3. $MnCl_2$ showed the ability to induce an increase in MP at a concentration of 12.6 μg/plate ($p<0.001$ between MP values for $MnCl_2$ (12.6 μg) treated and medium control cells), which was equivalent to the level of Mn^{++} that would be found on the UICC amosite concentration of 50 μg/ml. All of the lower concentrations of $MnCl_2.4H_2O$ also showed the ability to induce a cell membrane hyperpolarisation ($p<0.02$ between MP and IR values for $MnCl_2.4H_2O$ (5.8, 2.9 and 0.7 μg) treated and medium control cells). In order to establish if serum had a protective effect, $P388D_1$ cells were exposed to $MnCl_2.4H_2O$ (12.6 μg/plate) in the presence of conditioned medium (Table 5.4). The MP and IR values were recorded at regular intervals over a 3 hr time period; a maximum value was obtained for the hyperpolarising activity 60 mins following treatment ($p<0.001$ between both MP and IR values for $MnCl_2$ treated (60 mins) and medium control (90 mins) $P388D_1$ cells); after 60 mins the MP and IR values declined until the control levels were achieved.

Table 5.3 The Response of $P388D_1$ Cells One Hour Following Treatment With Various Concentrations of $MnCl_2.4H_2O$ in the Absence of Serum.

a) 12.6 µg/plate	MP (mV)	IR (MΩ)
Initial control	10.2 ± 0.9	34.0 ± 7.6
$MnCl_2.4H_2O$	15.9 ± 3.1	34.4 ± 16.2
Medium control	10.4 ± 1.0	28.7 ± 7.7
b) 5.8 µg/plate		
Initial control	9.5 ± 1.0	25.8 ± 7.9
$MnCl_2.4H_2O$	16.5 ± 2.1	45.8 ± 9.3
Medium control	10.4 ± 1.4	22.9 ± 2.9
c) 2.9 µg/plate		
Initial control	10.7 ± 1.7	20.5 ± 3.9
$MnCl_2.4H_2O$	14.6 ± 2.6	35.2 ± 14.1
Medium control	10.6 ± 1.0	22.9 ± 5.6
d) 0.7 µg/plate		
Initial control	11.1 ± 1.7	23.3 ± 2.4
$MnCl_2.4H_2O$	22.0 ± 4.8	36.7 ± 7.5
Medium control	13.7 ± 2.1	22.8 ± 3.5

Each value is a mean of 10 readings ± SD.

Table 5.4 The Response of $P388D_1$ Cells at Various Time Points Following Treatment With $MnCl_2.4H_2O$ (12.6 μg/plate) in the Presence of Serum.

Treatment	Time Point (mins)	MP (mV)	IR (MΩ)
Initial control	0	10.2 ± 0.9	34.0 ± 7.6
$MnCl_2.4H_2O$	20	12.9 ± 1.9	37.0 ± 6.5
	40	11.6 ± 1.1	54.9 ± 14.0
	60	16.2 ± 2.3	60.0 ± 19.3
	90	15.9 ± 2.5	55.2 ± 33.9
	120	15.5 ± 3.0	40.3 ± 9.9
	240	10.3 ± 0.7	24.4 ± 3.2
Medium control	90	12.2 ± 2.2	38.0 ± 11.2

Each figure is a mean of 10 readings ± SD.

5.3.6 An Investigation Into the Role of the Calcium Sensitive Potassium Channel in the Induction of the Cell Membrane Hyperpolarisation.

Before undertaking this investigation it was necessary to establish that the $P388D_1$ cell membrane possessed Ca^{++} sensitive K^+ channels. The cells were exposed to acebutalol which activates this particular conductance channel (MO Wright, personal communication) (Table 5.5). 10 mins following initial exposure to acebutalol (40 μg/ml), a significant increase in the cell MP was observed ($p<0.001$ between MP values for acebutalol-treated and initial control cells), thereby demonstrating that these cells possess Ca^{++} sensitive K^+ conductance channels. It was also shown that treating $P388D_1$ cells with quinidine-HCl (20 μM solution) prior to the addition of acebutalol effectively blocked the activation of this channel (Table 5.5), and it can therefore be concluded that quinidine is a useful agent to use for the prevention of activation of the Ca^{++} sensitive K^+ conductance channel.

Following this experiment, $P388D_1$ cells were exposed to quinidine for 10 mins to block the Ca^{++} sensitive K^+ conductance channel, they were then exposed to either $MnCl_2$ or UICC amosite for either 30 or 60 mins respectively (Table 5.6). Whilst $MnCl_2$ and UICC amosite effectively induced a cell membrane hyperpolarisation in the absence of quinidine ($p<0.05$ between both MP and IR values for either $MnCl_2$ or UICC amosite treated and 90 mins medium control cells); pre-exposure of $P388D_1$ cells resulted in the abolition of the hyperpolarisation induction by either of these agents, thereby suggesting that both Mn^{++} and UICC amosite cause a cell membrane hyperpolarisation by activating the Ca^{++} sensitive K^+ channel.

Table 5.5 The Response of P388D$_1$ Cells Following Treatment With Acebutalol, Quinidine, or Quinidine Followed by Acebutalol in the Absence of Serum.

Treatment	MP (mV)	IR (MΩ)
Initial control	11.1 ± 0.9	4.6 ± 2.9
Medium control for 10 mins	13.8 ± 5.1	6.2 ± 4.1
Acebutalol (40 μg/ml) for 10 mins	21.8 ± 5.1	8.0 ± 4.4
Quinidine-HCl (20 μM solution) for 10 mins	12.0 ± 1.8	5.4 ± 2.9
Quinidine-HCl (20 μM solution) for 10 mins followed by Acebutalol (40 μg/ml) for 10 mins	11.8 ± 1.9	3.8 ± 1.6

Each value is a mean of 10 readings ± SD.

Table 5.6 The Effect of Quinidine on P388D$_1$ Cell Manganese and Amosite-Induced Hyperpolarisations.

Treatment	MP (mV)	IR (MΩ)
Initial control	13.7 ± 4.6	9.4 ± 5.9
Quinidine (20 μM solution) for 15 mins	12.0 ± 3.4	4.2 ± 1.0
$MnCl_2.4H_2O$ (12.6 μg/plate) for 30 mins	19.8 ± 5.8	5.8 ± 2.4
Quinidine for 10 mins followed by $MnCl_2.4H_2O$ for 30 mins	13.6 ± 3.4	6.4 ± 4.8
UICC amosite (50 μg/ml) for 60 mins	24.9 ± 11.9	6.4 ± 2.6
Quinidine for 10 mins followed by UICC amosite for 60 mins	13.7 ± 3.0	4.1 ± 3.5
Medium control for 90 mins	15.0 ± 2.5	5.1 ± 5.1

Each value is a mean of 10 readings ± SD.

5.4 DISCUSSION.

The study of the electrophysiological properties of the macrophage membrane has recently acquired considerable attention because of the important role played by the macrophage membrane in the initiation of a variety of interactions in the body. It has been shown that the onset of a number of these interactions can involve a considerable perturbation of the membrane electrophysiology, and modifications of both the MP and IR have been noted during the initial stages of macrophage functions eg phagocytosis (Kouri et al, 1980), chemotaxis (Gallin and Gallin, 1977; Gallin et al, 1980) and cellular activation following exposure to lymphokine (Niemtzow et al, 1979). These types of studies have been extended to include the examination of the effect of various types of mineral particles and fibres on the electrophysiology of the $P388D_1$ cell membrane (Gormley et al, 1978; Gormley and Wright, 1980; Wright and Gormley, 1980), and these studies proved interesting as the different types of dust samples exerted different modulatory effects on the electrical properties of the cell membrane. It was therefore suggested that further studies with regard to dust-induced alterations in cell membrane electrophysiology would prove useful adjuncts to the conventional methods of cytotoxicity analysis, and results from such experiments could prove instrumental in the elucidation of the mechanism of dust/cell interactions (Gormley and Wright, 1980; Wright and Gormley, 1980). One of the major aims of this study was therefore to establish the usefulness of this technique for the examination of fibre/cell interactions, as well as to investigate the electrophysiological response of $P388D_1$ cells to treatment with different fibrous samples.

5.4.1 The Electrophysiological Properties of the $P388D_1$ Cell Membrane and Their Modification Following Treatment with UICC Asbestos.

One of the major problems encountered by those research groups endeavouring to assess the electrophysiological properties of the macrophage membrane is the accurate insertion of the ME into the cells without the induction of cell damage. In general, macrophages are small cells, hence the problems encountered upon ME insertion, but

this situation has been overcome to some extent by culturing the cells for periods of up to 3 or 4 weeks *in vitro* to encourage maturity and hence a greater cell size (Gallin and Gallin, 1977),or alternatively the cells may be obtained as large and activated cells following intraperitoneal injection of dextran (Persechini *et al*, 1981), mineral oil (Gallin *et al*, 1975) or thioglycollate broth (Gallin and Livengood, 1980); macrophage polykarya were utilised by Persechini *et al* (1981). The use of macrophage populations elicited by different biochemical means would, however, involve those problems normally associated with the use of populations of cells which are in different activational states, in that the cells may respond differently depending upon their activational status, and the question arises as to the choice of the eliciting agent. Chapter 4 of this thesis describes how the activational problem was surmounted in this study by employing the macrophage-like $P388D_1$ cell. Gormley *et al* (1978) compared the electrophysiological properties of guinea pig alveolar macrophages and $P388D_1$ cells following treatment with different particulate samples, and both types of cell showed a similar response to a particular dust. The $P388D_1$ cells proved advantageous because of their ready availability, and also their larger size ie 17.8 μm diameter for $P388D_1$ cells and 11.2 μm for guinea pig alveolar macrophages (Gormley *et al*, 1978). It was therefore decided that the $P388D_1$ cell line would be a useful phagocytic substitute for the macrophage in this particular study.

The routine measurement of the MP and IR values for the $P388D_1$ cells proved straightforward and there were no problems associated with ME insertion in this study. The mean resting MP values for the cells tended to vary between experiments ie from -6.5 to -13.8 mV, and a similar trend was noted for the IR values ie from 5 to 34 MΩ. Previous work on $P388D_1$ cell membrane electrophysiology has also resulted in a slight discrepancy regarding the archival resting MP values; initial studies by Gormley *et al* (1978) recorded -6.4 mV for control cells, but a value of -9.5 mV was noted in a later study (Wright and Gormley, 1980). These differences were ascribed to the inavoidable slight variations in cell density (Wright and Gormley, 1980), and this explanation may account for the observed variations in this study. In general, however, the MP values obtained for the $P388D_1$ cells

were similar to those observed for primary macrophages (Gallin et al, 1975; Gallin and Gallin, 1977; Gormley et al, 1978; Persechini et al, 1981). Gallin and Livengood (1980) reported that their macrophage cultures (thioglycollate-induced mouse peritoneal macrophages) contained cells which had one of two possible resting potential values; the majority of cells had MP values in the range of -20 to -40 mV, however a small proportion had high MP values of -60 to -90 mV, and it was suggested that the macrophages may be able to flip from one state to the other, depending on a number of contributing factors such as macrophage activation and membrane fluidity. No evidence for the existence of two resting potential states was observed in the $P388D_1$ cell cultures, although the occurrence of an occasional spontaneous hyperpolarisation, similar in nature to those recorded by Gallin et al (1975) and Persechini et al (1981) was observed. A recent study by Ince et al (1983) has shown that the $P388D_1$ cell and macrophage MP values may be at least twice as negative as the sustained potential values previously reported. The authors (Ince et al, 1983) showed that the initial insertion of the ME into $P388D_1$ cells resulted in a peak value of -37 mV within 2 msec, the potential then depolarised to -12 mV in 20 msec and slowly repolarised to give a sustained potential of -14 mV. The effect noted by Ince et al (1983) has not been observed in this study; comparisons between the two studies are difficult, however, as Ince et al (1983) used potassium acetate to fill their electrodes, rather than the KCl used in this study, thereby creating electrodes of different resistance values.

Modifications of the electrophysiological properties of the $P388D_1$ cell membrane during exposure to various types of UICC asbestos are described in Section 5.3.2 of this chapter. A similar response was obtained to the one described by Wright and Gormley (1980) and Gormley and Wright (1980). UICC crocidolite had no significant effect on either cell MP or IR regardless of whether the treatment involved the inclusion or exclusion of serum. The results obtained using UICC crocidolite were consistent with the viability data, as no evidence for cell death was obtained; a reduction in the number of live cells per field was noted at the 48 hrs time point compared to the appropriate control, but this could be attributed to a reduction in cell division rather than cell death as the number of viable cells counted did not

fall below the level obtained for the initial control. The treatment of $P388D_1$ cells with UICC amosite (Figure 5.14 and 5.17) resulted in the occurrence of a cell membrane hyperpolarisation at the 1 hr time point, and the hyperpolarisation occurred in either the presence or absence of serum. This finding was also recorded by Gormley and Wright (1980), who, in addition, noted a decrease in the MP and IR values at the 48 hrs time point (Figure 5.17). The apparent reduction in MP and IR was observed in this study, although the effect was not accompanied by a reduction in cell viability. The assessment of the cell membrane electrophysiological properties is reputedly a more sensitive indicator of cell membrane damage than the trypan blue exclusion assay (Stephens and Henkart, 1979), and it is therefore feasible that membrane damage due to amosite exposure was being detected in the electrophysiological assay prior to detection in the trypan blue assay.

The treatment of $P388D_1$ cells with UICC chrysotile (Figure 5.15 and 5.18) resulted in a decrease in both MP and IR values 24 hrs after treatment in the presence of serum; a corresponding decrease in cell viability was also noted which was consistent with previous reports regarding the cytotoxic nature of this fibrous sample on cell electrical properties (Gormley and Wright, 1980; Wright and Gormley, 1980). Wright and Gormley (1980) examined the cytotoxic nature of UICC chrysotile and found that the addition of serum effectively reduced the speed with which both MP and IR values decreased, hence delaying the onset of cytotoxicity. In this study, however, the observed MP and IR values for up to 6 hrs following treatment with UICC chrysotile were similar, regardless of whether serum was present or not; thus no evidence was obtained to suggest that serum was exerting a protective effect against the cytotoxic action of chrysotile. Allison (1971) showed that chrysotile could kill macrophages within 2 hrs following exposure in the absence of serum, however the presence of serum protected the cells for 48 hrs. In this study, a concentration of 4% serum was used rather than the 10% utilised by Allison (1971), and thus the cell death observed at 24 hrs following exposure to chrysotile may be expected. However, the lack of "early cytotoxicity" in the absence of serum is inexplicable, and possibly demonstrates a difference in the susceptibility of the $P388D_1$ cell membrane to the action of cytotoxic

dusts compared to the macrophage. The discrepancy between the results obtained in this study and those noted by Wright and Gormley (1980) can only be explained in terms of a slight change in the membrane characteristics of these cells due to increased routine passaging in tissue culture conditions.

In general this study has shown that the assessment of the electrophysiological properties of the $P388D_1$ cell membrane during exposure of the cells to a dust sample is a sensitive method of assessing membrane damage. However, when considering the incorporation of this assay into a routine toxicological system, several disadvantages were found to be associated with its use: i) the apparatus required to obtain electrophysiological recordings is highly specialised and therefore expensive; ii) the physical limitations regarding the practical application and operator use of the equipment allows the assessment of one plate only per treatment condition, and it is therefore not possible to establish replicates in this assay system; iii) similar limitations also allowed the assessment of 10 cells only per plate, which is a very small proportion of the whole plate; iv) the system was subject to operator bias because it was necessary that each individual cell examined should be chosen by the operator. The information gained from the electrophysiological measurements did not improve upon the conventional trypan blue exclusion or enzyme release assays, despite the possibility of superior sensitivity. It must therefore be concluded that the measurement of electrophysiological properties is not an ideal assay system for inclusion in a routine toxicological test system. The assay did, however, prove useful for identification of unusual membrane perturbing effects induced following exposure to dust, in particular the hyperpolarising effect observed during treatment with UICC amosite was of interest and warranted further attention. A detailed dissection of the amosite-induced plasma membrane hyperpolarisation will be discussed in the following section.

5.4.2 The $P388D_1$ Cell Membrane Hyperpolarisation and Its Induction Following Exposure to Amosite Fibres.

In order to determine whether the hyperpolarising capacity was common to all amosite samples, $P388D_1$ cells were exposed to amosite

fibres from different sources. E UICC amosite and E LF amosite induced a hyperpolarisation 1 hr after their addition to $P388D_1$ cells; E F amosite required 2 hrs to induce a hyperpolarisation, but this effect was complicated by the presence of contaminating elements from the factory environment. SF amosite, however, did not elicit any response during the 6 hrs of exposure. SF amosite was prepared from the LF amosite sample by grinding and sedimentation in water (JMG Davis, personal communication), and it must therefore be considered that the hyperpolarising agent may have been removed from the surface of the fibres during the preparation procedure. In order to examine this possibility, samples of amosite were leached in culture fluid for various periods of time; these samples were not, however, subjected to grinding. After 25 days of leaching it was found that the hyperpolarising activity of the fibres had been completely removed (Table 5.2), and the supernatant leachate had acquired the ability to induce a cell membrane hyperpolarisation. The agent causing the hyperpolarisation may therefore have been loosely bound to the surface of the amosite fibre, and may be a contaminating material rather than an integral part of the fibrous structure. A report on the levels of contaminating ions found on UICC asbestos fibres following a study by Morgan and Cralley (1973) is described in Table 1.2. The ion Mn^{++} was found on UICC amosite fibres at a level of 13000 ppm , whereas UICC crocidolite and chrysotile showed levels of only 140 and 231 ppm respectively (Morgan and Cralley, 1973). The addition of the appropriate concentration of Mn^{++} in the form of $MnCl_2.4H_2O$ to cultures of $P388D_1$ cells resulted in the induction of a cell membrane hyperpolarisation which reached a peak value after 1 hr of incubation (Table 5.4). The addition of quantities of Mn^{++} which were considerably less than would be found on the surface of UICC amosite fibres also caused a significant hyperpolarisation (Table 5.3). The addition of serum to the medium did not prevent the hyperpolarising activity of Mn^{++}.

This type of hyperpolarising activity has been observed in macrophage cultures following the intracellular injection of Ca^{++} (Persechini *et al*, 1981), the use of Ca^{++} ionophores A23187 and X537A (Oliveira-Castro and Dos Reis, 1981), and stimulation with chemotactic factors (Gallin and Gallin, 1977). On the basis of experimental evidence, the

hyperpolarisation was attributed to a rapid K^+ efflux due to stimulation of the Ca^{++} sensitive K^+ channel (Persechini et al, 1981; Oliveira-Castro and Dos Reis, 1981; Gallin et al, 1975; Gallin and Gallin, 1977). It must therefore be considered that Mn^{++} and/or amosite may induce the membrane hyperpolarisation by stimulating this particular conductance channel. Before testing this hypothesis, however, it was necessary to demonstrate the presence of the Ca^{++} sensitive K^+ channel in the $P388D_1$ cell membrane. Propanolol, a β-adrenoreceptor blocker, has the ability to activate this channel (Gardos et al, 1975), and acebutalol, another β-adrenoreceptor blocker (Basil et al, 1973, 1974) also has this property (MO Wright, personal communication). The exposure of $P388D_1$ cells to acebutalol resulted in the induction of a membrane hyperpolarisation (Table 5.5). Quinidine, which has an inhibitory effect on the Ca^{++} induced K^+ loss via this channel (Armando-Hardy et al, 1975), was effective in blocking the action of acebutalol on $P388D_1$ cells (Table 5.5). These results illustrate that there are Ca^{++} sensitive K^+ conductance channels in the $P388D_1$ cell membrane. The pre-exposure of $P388D_1$ cells to quinidine resulted in the inhibition of both the Mn^{++} and amosite induced hyperpolarisation (Table 5.6), thereby indicating that Mn^{++} and amosite induced a hyperpolarisation in the $P388D_1$ cell membrane by stimulating the Ca^{++} sensitive K^+ channel. Akaike et al (1981) and Hagiwara (1981) have shown that the Ca^{++} channel is permeable to Mn^{++}; the entry of Mn^{++} into the Ca^{++} channel may therefore result in a rapid efflux of K^+ via the K^+ channel. As to whether or not Mn^{++} is the contaminating agent responsible for the amosite-induced hyperpolarisaton has not been confirmed in this study; a further chemical analysis of asbestos fibres and leachate supernatants may result in clarification of this situation.

The possibility that an ionic component of asbestos, such as Mn^{++}, can severely perturb the cell membrane is of interest. It is known that macrophages treated in vivo with asbestos, whilst possessing a number of activational characteristics such as increased spreading on glass, increased Fc receptor avidity, do not, however, possess a tumouricidal capacity (Donaldson et al, 1982). Heavy metal ions such as cadmium can also inhibit the tumouricidal activity of macrophages by interfering with specific ionic sites on the cell membrane (Nelson et al, 1982). It is feasible therefore, that certain ionic components

of the asbestos fibre may interfere in a similar manner with the macrophage membrane such that interaction with other cells is suppressed. A further study of various ionic components of asbestos fibres on the macrophage membrane electrical properties, together with an examination of any corresponding immunosuppressant action may therefore prove fruitful in clarifying their mode of action.

The examination of the cell membrane electrophysiological properties during the interaction of $P388D_1$ cells with various asbestos samples, whilst proving unsuccessful for use in a routine toxicological assay has proven to be particularly useful for examining the mechanism of dust/cell interactions. It is hoped therefore, that a further study comprising the examination of the effects of various conductance channel inhibitors on asbestos-induced cytotoxicity will help to elucidate the mechanism by which certain dust samples exert their cytotoxic sequelae.

5.5 CONCLUSIONS.

This study has involved an examination of the asbestos-induced modifications of the $P388D_1$ cell membrane electrophysiological properties. The more cytotoxic asbestos samples caused an earlier decline of both MP and IR values than the less cytotoxic samples. The amosite samples were unique in their ability to induce a cell membrane hyperpolarisation, which was shown to be due to activation of the Ca^{++} sensitive K^{+} channel. This assay system did not prove to be useful for inclusion in a routine toxicological screening system, but was of considerable use for the examination of the mechanism of fibre/cell interaction.

CHAPTER 6 THE CYTOTOXIC ACTIVITY OF ASBESTOS TOWARDS POPULATIONS OF MACROPHAGES IN DIFFERENT STATES OF ACTIVATION.

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CHAPTER 6 THE CYTOTOXIC ACTIVITY OF ASBESTOS TOWARDS POPULATIONS OF MACROPHAGES IN DIFFERENT STATES OF ACTIVATION.

6.1 INTRODUCTION.

The morphological and functional properties of the macrophage have maintained considerable interest within the research world for a number of years. Extensive studies regarding these properties have recently shown, however, that many populations of macrophages are heterogeneous in nature, and the degree of heterogeneity may vary depending upon the source of the cells and also the nature of any prior stimulating agents to which they may have been exposed (see review by Hopper et al, 1979). The major factor that contributes to the heterogeneity both within and between populations of macrophages is the activational status of the individual cells themselves and hence the relative proportion of activated macrophages within each population. The properties of an unstimulated macrophage vary considerably from those of an activated macrophage (see Table 1.7) and those features associated with cellular activation include an increase in cell size (Hopper et al, 1979), surface receptors (Rhodes, 1975), acid hydrolase and neutral protease secretion (Davies and Bonney, 1980) and enhanced anti-microbial and anti-tumour activity (Nathan, 1982).

The ability of asbestos fibres to modify various macrophage functions has also attracted considerable interest, and in particular the ability of chrysotile to induce an increase in the release of lysosomal hydrolases has warranted attention because of the association between lysosomal enzyme secretion and fibrosis (Davies and Allison, 1976). A number of research groups have shown that chrysotile can induce a selective release of lysosomal hydrolases (Davies et al, 1974a; Morgan et al,1977; Jaurand et al , 1980a); however a number of conflicting reports have also been published, as Jaurand et al (1978) and Davies (1980b) noted some loss of LDH upon exposure of macrophages to chrysotile, similarly Kang et al (1979) observed a loss of another cytoplasmic enzyme phosphohexose isomerase under similar conditions. Miller (1978) and Morgan and Allison (1980) have given these differences some consideration and suggested that populations of macrophages

which have been stimulated by different biochemical agents in vivo may respond in a differing manner in vitro following exposure to dust. Certainly, a variety of different agents have been used to elicit populations of macrophages and also macrophages from different sites in the body of various animal species have been employed, for example unstimulated rabbit alveolar macrophages (Jaurand et al, 1980a), proteose peptone elicited mouse peritoneal macrophages (Davies et al, 1974a), unstimulated mouse peritoneal macrophages (Davies, 1980a,b), and even the $P388D_1$ macrophage-like permanent cell line (Wade et al, 1976; Wright et al, 1980). It would therefore be expected that populations of macrophages from different sources that have been elicited by different agents would possess modified biochemical properties (Hopper et al, 1979) and may respond in a diverse manner to treatment with samples of dust. The alveolar macrophage is constantly exposed to activating agents such as inhaled bacterial and mycotic pathogens, and it would be of some importance to examine the response of pre-activated macrophages to treatment with asbestos fibres. A recent report has shown that macrophages become activated following in vivo exposure to asbestos fibres (Donaldson et al, 1982), and it would therefore be relevant to establish the susceptibility of asbestos-activated macrophages to the cytotoxic activity of the various asbestos types.

6.1.1 Aims and Objectives.

The main aims of this study were:

i) To produce populations of macrophages elicited following intra-peritoneal injection of different agents in vivo, and to assess their activational status.

ii) To examine the response of each population of macrophages to treatment with UICC crocidolite, amosite and chrysotile; the trypan blue exclusion test and LDH levels would be used to assess membrane permeability, and glucosaminidase would demonstrate lysosomal enzyme loss.

iii) In addition, the electrophysiological properties of each population would be examined to establish the integrity of the cell membrane and to demonstrated any alterations in MP and IR values that may be due to the activational status of the cell.

6.2 MATERIALS AND METHODS.

6.2.1 Experimental Rationale.

The aim of this study was to examine the cytotoxic effect of asbestos fibres *in vitro* on macrophages in different activational states. The question therefore arose regarding the source of the macrophages to be studied. Mice were employed because of their relatively low cost, both to buy and maintain, and also because of their ready accessibility. Ideally, the most relevant macrophage to study would have been the alveolar macrophage; however a preliminary study by the author demonstrated that lavage of the mouse lung resulted in a cell harvest of 2×10^5 macrophages only, whereas lavage of the peritoneal cavity resulted in a yield of 5×10^6 macrophages. The use of the peritoneal cavity therefore appeared to be superior in order to obtain sufficient cells to establish the various cytotoxicity assays. Morgan and Allison (1980) have shown that the alveolar macrophage responds to dust treatment in a similar manner to the peritoneal macrophage; the peritoneal cell would therefore be a reasonable substitute for the alveolar cell.

6.2.2 Mice.

Twelve week old, male, specific pathogen free CBA mice were used throughout the study. The animals were maintained on normal laboratory diet and water; both were supplied *ad libitum*.

6.2.3 In Vivo Treatment of Mice With Various Stimulating Agents.

In order to obtain populations of peritoneal macrophages in a variety of activational states, groups of mice were inoculated intra-peritoneally with one of the following agents:

i) No treatment ie untreated control.
ii) 1 ml sterile phosphate buffered saline (PBS) (Gibco Biocult).
iii) 1 ml 10% sterile proteose peptone (Difco Bacto) in distilled water.
iv) 1.4 mgs of heat-killed *Corynebacterium parvum* (*C parvum*) (Wellcome) in 0.2 mls PBS.

v) 1 ml of 0.1% latex beads (0.81 μm diameter) (Difco Bacto) in sterile PBS.

vi) 1 ml of sterile PBS containing 2.5 mgs of UICC crocidolite.

vii) 1 ml of sterile PBS containing 2.5 mgs of UICC amosite.

viii) 1 ml of sterile PBS containing 2.5 mgs of UICC chrysotile.

The injections were carried out in the absence of anaesthesia using a 23 guage needle, excepting for the three asbestos samples for which a 21 guage needle was substituted to avoid needle blockage. At least three animals were inoculated for every treatment group in each experiment.

6.2.4 Harvesting of Mouse Peritoneal Macrophages.

Three days following intraperitoneal inoculation, the mice were sacrificed using an over-dose of ether. The abdominal area of each mouse was washed with 70% alcohol and dried. The abdominal skin and muscles were reflected and the peritoneal cavity injected with 5 mls of F10 medium (Gibco-Biocult) containing 20% heat-inactivated foetal calf serum(FCS) (Gibco-Biocult) (complete medium). The peritoneal wall was massaged to encourage the peritoneal exudate cells (PECs) to suspend in the medium. The medium containing cells was withdrawn via a 19 gauge needle into a 10 ml syringe and stored in a sterile, siliconised glass universal container on ice. The PECs from those animals within each treatment group were pooled.

The pooled PECs were centrifuged for 10 mins at 200g ; the supernatant was removed and the cells resuspended in complete medium. The cell number was assessed using an improved Neubauer Haemocytometer and the volume adjusted to give the required cell concentration. Cell viability was determined using trypan blue (for method see Section 4.2.3.1). Any suspensions in which less than 95% of the cells were viable were discarded.

6.2.5 Spreading Assay to Assess the Degree of Activation of Macrophage Populations.

A spreading assay was employed to determine the activational status

of each population of macrophages (Donaldson et al, 1982). 0.1 mls of complete medium containing 1 x 10^5 PECs were placed on glass coverslips (6 x 22 mm) (Chance Propper) and incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. After precisely 1 hr the coverslips were washed vigorously to remove any non-adherent cells. The remaining adherent macrophages were fixed and stained (for methods see Section 4.2.5) and mounted in DPX (Gurr). 100 cells from each coverslip were scored according to their ability to i) spread completely on glass, ii) adhere to the glass but remain unspread. Triplicate coverslips were assessed for each population of cells. The relative degree of activation of each population was expressed in terms of the percentage of cells completely spread.

6.2.6 In Vitro Culture and Treatment of Peritoneal Exudate Cells.

3 mls of complete medium containing 1 x 10^6 viable PECs were seeded in a 3.5 mm tissue culture dish (Nunc). The plates were placed on trays and these were rocked from side to side to encourage an even dispersal of the cells. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 1 hr the cell cultures were washed vigorously with PBS to remove non-adherent cells and the dishes were replenished with 3 mls of complete medium. The cultures were then treated as follows:

6.2.6.1 The Assessment of the Phagocytic Capacity of the Peritoneal Exudate Cell Populations and Their Viability Following Phagocytosis.

In order to assess the phagocytic capacity of each PEC population, three dishes for each population were treated with 30 µl of concentrated latex bead solution (0.81 µm in diameter) (Difco Bacto), and incubated at 37 °C. After 24 hrs each plate was washed with PBS to remove excess beads, and examined microscopically at a magnification of x 100. Five randomly chosen fields of view were examined for each plate, and the cells scored according to their ability to phagocytose the beads (a cell was termed phagocytic if it contained more than 3 latex beads). The 5 scores for each plate were amalgamated and expressed in terms

of the percentage of phagocytic cells per plate. In order to assess the viability of each population of PECs following phagocytosis of beads, the phagocytic cells were also scored for their ability to exclude trypan blue. The 5 scores for each plate were amalgamated and expressed in terms of percentage of viable cells per plate.

6.2.6.2 Treatment of Peritoneal Exudate Cells With Asbestos Fibres.

10.0 mg samples of UICC crocidolite, amosite and chrysotile A were prepared as described previously (Section 2.2.4.2) by ultrasonication for 2 mins in 10 mls of complete medium. The plates were treated with one of the following:

i) No treatment ie untreated control.
ii) 100 µl of medium containing 100 µg of UICC crocidolite.
iii) 100 µl of medium containing 100 µg of UICC amosite.
iv) 100 µl of medium containing 100 µg of UICC chrysotile A.

The plates were agitated to ensure an even dispersal of the dust and the plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 24 and 48 hrs the cytotoxicity assays were performed.

6.2.7 Cytotoxicity Assays.

6.2.7.1 Cell Viability.

After 24 and 48 hrs cell viability was assessed using the trypan blue exclusion test (Section 4.2.3.5a). 5 randomly chosen fields of view were examined for each plate, and the cells scored according to their ability to exclude trypan blue. The 5 values obtained for each plate for the number of viable cells were presented as a mean value. The overall percentage for each treated plate was calculated:

$$\text{Percentage viability} = \frac{\text{number of live cells/treated plate}}{\text{number of live cells/untreated control plate}} \times 100.$$

6.2.7.2 Biochemical Assays.

a) Harvesting of Cells.

24 hrs following treatment with asbestos, the culture medium and cells were harvested from each plate according to the method described in Section 4.2.3.5c , with the exception that the cells were lysed with 1 ml of Triton-X solution.

b) LDH Assay.

The levels of LDH present in the culture medium and the cells were assessed (for method see Section 4.2.3.5d). The factors used to convert the absorbance value to enzyme units were adjusted to account for the alteration in the volumes of both medium and cell lysate, thus the cell conversion factor became 1.266, and the medium factor 3.798.

c) Glucosaminidase Assay.

The levels of the glucosaminidase present in the culture medium and the cell lysate were assessed (for method see Section 4.2.3.5e). As in the case of the LDH assay the factors used to convert the absorbance values to enzyme units were adjusted; the medium factor became 0.0115 and the cell lysate factor became 0.0346.

The enzyme levels for each treatment condition were finally expressed as the percentage of the total enzyme activity released into the medium per plate.

6.2.8 The Activation of Macrophages In Vitro With Lymphokine and Their Response to Treatment With Chrysotile.

In order to provide populations of macrophages that had been activated in vitro it proved necessary to select an appropriate activating agent. A sample of lymphokine (a macrophage-activating agent) was used, and this was prepared and donated by K Donaldson (IOM, Edinburgh).

6.2.8.1 Preparation of Lymphokine.

The lymphokine was prepared according to the method of Lazdins et al (1978). Mouse (CBA) spleens were taken, and placed in a glass vial with 1 ml of F10 medium. The splenocytes (mixed lymphocytes) were dispersed using a glass plunger. The suspended cells were passed through a nylon wool column to remove B lymphocytes, and the resulting T lymphocytes were harvested and adjusted to a concentration of 1×10^6 cells/ml of F10 medium. The T lymphocytes were exposed to Concanavalin A (Con A) (Sigma) (10 μg/ml) at 37 °C. After 24 hrs the suspension was centrifuged at 200 g for 15 mins; the supernatant containing lymphokine was harvested and stored at -4 °C until required.

6.2.8.2 The Treatment of Macrophages With Lymphokine and UICC Chrysotile.

Saline-induced macrophages (Section 6.2.3) were harvested and plated out (for method see Sections 6.2.4 and 6.2.6). After 1 hr they were washed with PBS and then subjected to one of the following treatments:

i) Complete medium only.
ii) Lymphokine in complete medium.
iii) Complete medium supplemented with Con A (10 μg/ml).

The plates were incubated at 37 °C for 24 hrs to allow the macrophages to become activated. Three of the plates from each of conditions i), ii) and iii) were untreated, and 3 plates from each of i), ii) and iii) were exposed to UICC chrysotile A (0.1 mls containing 100 μg, for method see Section 6.2.6.2). After incubation for a further 24 hrs the viability of the cells was assessed (for method see Section 6.2.7.1). The cellular viability was expressed as follows:

$$\text{percentage viability} = \frac{\text{no of viable cells per test plate}}{\text{no of viable cells per appropriate untreated control}} \times 100$$

6.2.9 The Electrical Properties of the Cell Membranes of Macrophages in Different Activational States.

In order to assess further the integrity of the cell membranes of

the various populations of macrophages, the MP and IR values were established for each group of cells. The populations of macrophages were harvested and cultured as described previously (Section 6.2.4 and 6.2.6). Initially, problems were encountered when endeavouring to insert the ME into the macrophages, the cells were small and proved to be very thin after adherence and spreading onto the substrate had occurred. This situation contributed considerably towards the breakage of MEs and difficulty in maintaining MP and IR values for adequate measurement. It was found that supporting the macrophages on Thermonox coverslips (Flow) improved this situation because of the flexibility of the coverslip; the PECs were therefore routinely cultured on Thremonox coverslips supported in culture dishes. The cell MP and IR values for 10 cells from each population of macrophages were established.

6.2.10 Fixation, Staining and Photography.

The cells were fixed, stained and photographed as described previously (Section 4.2.5).

6.2.11 Statistical Analysis.

The Student T test was employed to demonstrated the statistical significance of any observed differences (Bailey, 1974).

6.3 RESULTS.

6.3.1 The Morphological Appearance of Macrophages Induced by Different Agents.

The morphological appearances of mouse peritoneal macrophages following induction *in vivo* by various agents and culture *in vitro* for 24 hrs are shown in Figures 6.1 to 6.5. The unstimulated, resident macrophages (Figure 6.1) whilst firmly adherent to the plastic substrate, remained rounded and did not spread to any great extent. The proteose peptone-elicited population of cells (Figure 6.2) displayed an enhanced ability to spread onto the plastic surface, and the macrophages became elongated and spindle-shaped; the *C parvum*- activated macrophages (Figure 6.3) however, whilst spreading efficiently in a "rounded" formation were highly vacuolated or "foamy" in appearance. Those populations of macrophages elicited following asbestos injection (Figures 6.4 and 6.5, UICC amosite not shown), were similar in morphological appearance to the *C parvum*- activated macrophages, and in some instances fibres could be seen in association with these cells. It should be noted that UICC chrysotile, despite its cytotoxic ability *in vitro*, had the capacity to induce populations of cells *in vivo* which possessed those morphological characteristics normally associated with healthy and viable cells. All of these populations of cells showed a high viability following isolation from the host mouse, in that greater than 95% of each population of cells showed the ability to exclude trypan blue.

6.3.2 The Degree of Activation of the Macrophage Populations.

The relative degree of activation of each macrophage population was expressed in terms of the proportion of cells within each population that possessed an enhanced ability to spread on glass (Figure 6.6). The untreated, saline and latex-elicited populations proved similar in their overall lack of ability to spread efficiently during one hour of incubation *in vitro*. The proteose peptone-elicited populations of PECs showed a slightly enhanced increase in spreading ability ($p<0.05$ between unstimulated and proteose peptone-induced populations of cells)

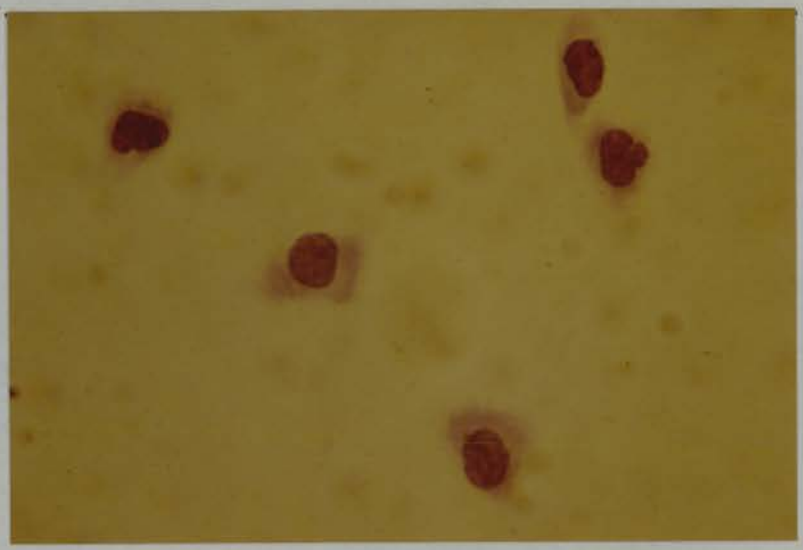

Figure 6.1 The Morphological Appearance of Unstimulated Mouse Peritoneal Macrophages 24 Hrs Following Culture In Vitro. (x 450).

Figure 6.2 The Morphological Appearance of Proteose Peptone-Induced Macrophages 24 Hrs Following Culture In Vitro. (x 450).

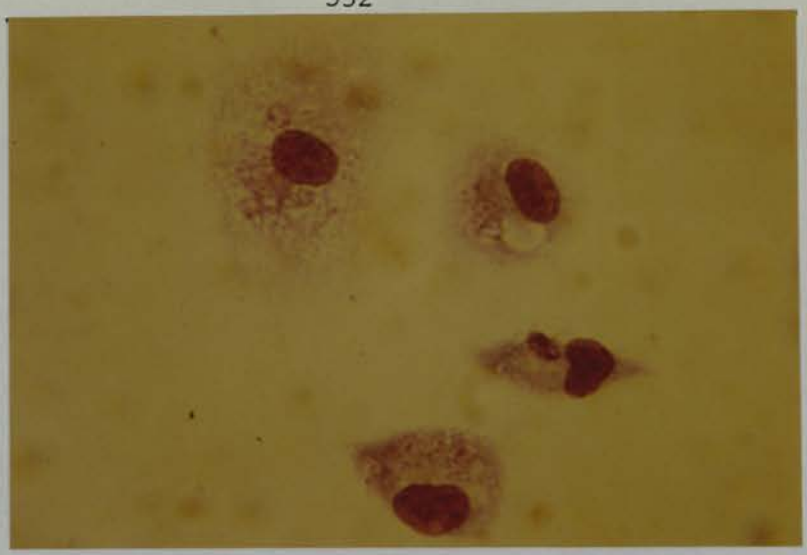

Figure 6.3 The Morphological Appearance of C parvum-Activated Macrophages 24 Hrs Following Culture In Vitro. (x 450).

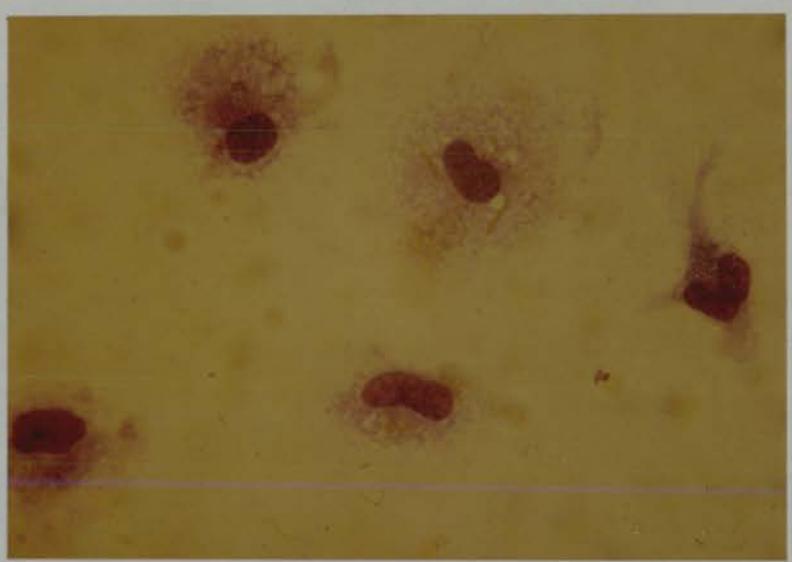

Figure 6.4 The Morphological Appearance of UICC Crocidolite-Elicited Macrophages 24 Hrs Following Culture In Vitro. (x 450).

Figure 6.5 The Morphological Appearance of UICC Chrysotile-Elicited Macrophages 24 Hrs Following Culture In Vitro. (x 450).

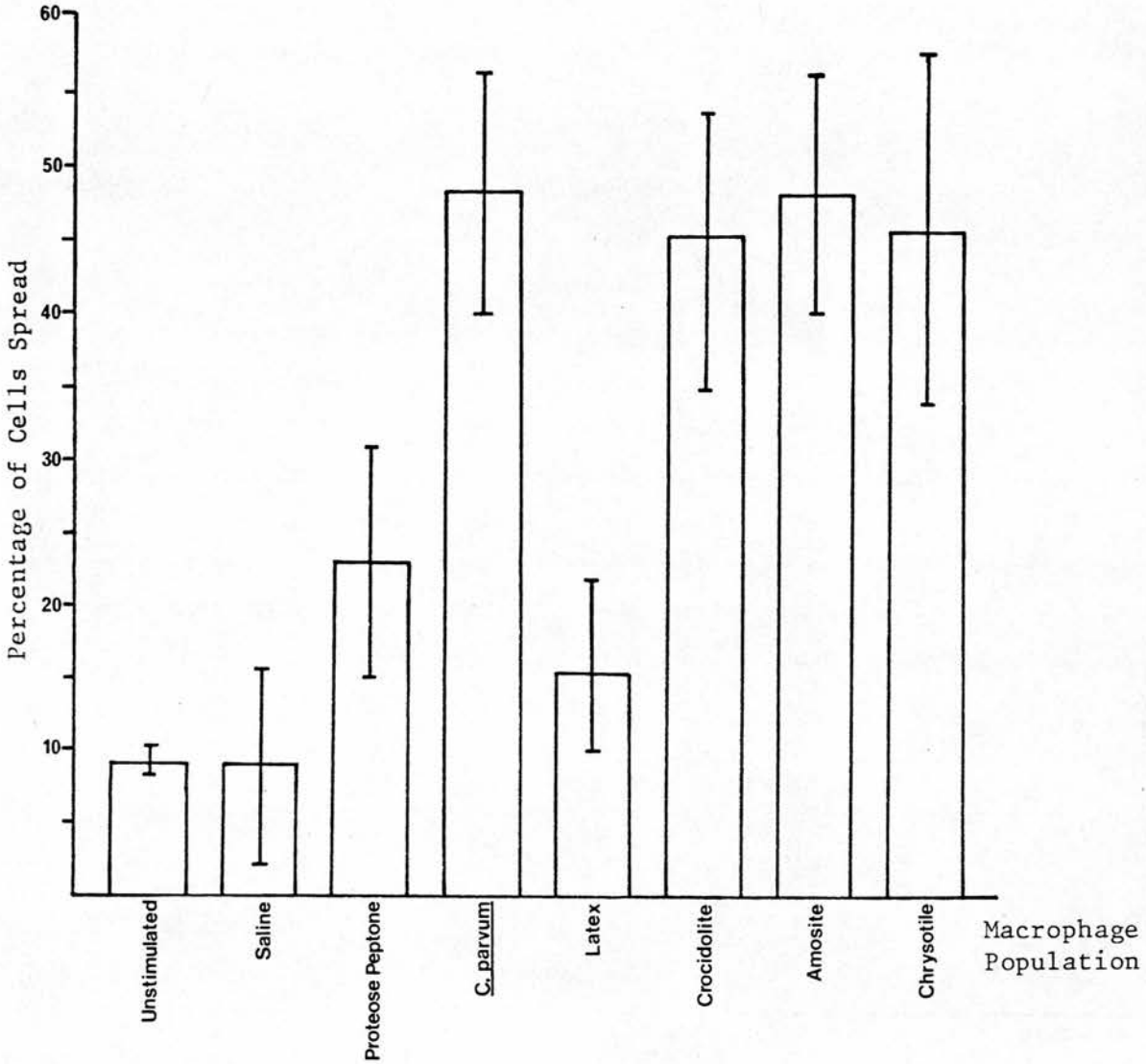

Figure 6.6 Relative Degree of Activation of Macrophage Populations According to Spreading Assay.

Each figure is a mean of 3 experiments ± SD.

thereby indicating an enhanced degree of activation according to this method of assessment. The C parvum-elicited macrophages proved to be even more activated than the proteose peptone-induced cells ($p<0.02$ between C parvum and proteose peptone-elicited populations of cells). The UICC crocidolite, amosite and chrysotile-induced populations of macrophages displayed a similar degree of activation according to this method of assessment, and proved to be as activated as the C parvum induced macrophages ($p>0.1$ between C parvum, UICC crocidolite, amosite and chrysotile-induced populations of cells).

6.3.3 The Phagocytic Capacity and Viability of Macrophage Populations Following Treatment With Latex Beads.

In order to assess the phagocytic capacity of the macrophage populations, unstimulated, saline, proteose peptone, C parvum and chrysotile-induced cells were exposed to latex beads; after 24 hrs the number of cells associated with more than 3 beads was noted (Table 6.1). All of the populations of cells contained macrophages of which at least 93% were shown to be phagocytic. In addition, at least 94% of the phagocytic cells proved to be viable following ingestion of latex beads, thus illustrating that the phagocytic event alone did not result in cell death.

6.3.4 The Morphological Appearance of Macrophage Populations Following Treatment With UICC Crocidolite or Chrysotile A.

The morphological appearance of proteose peptone and UICC crocidolite-elicited populations of macrophages, 24 hrs following treatment with either UICC crocidolite or chrysotile (100 µg/plate) are shown in Figures 6.7 to 6.10. Those proteose peptone- induced cells that had been treated with UICC crocidolite or chrysotile (Figures 6.7 and 6.8) had a morphological appearance consistent with a relatively high viability, very little deterioration of the intracellular contents was noted, although an increase in cellular vacuolation was observed. The UICC crocidolite treated crocidolite-induced population also appeared viable (Figure 6.9), although the exposure of these cells to UICC chrysotile (Figure 6.10), resulted in the occurrence of cells that were densely stained and pyknotic, thereby suggesting cell death.

Table 6.1 Percentage of Phagocytic Cells and Percentage Cell Viability of Macrophage Populations After Incubation for 24 Hrs With Latex Beads.

In Vivo Treatment of Macrophage Population	% Cells Phagocytic	% Viability
Unstimulated	98 ± 1	99 ± 5
Saline	96 ± 1	106 ± 6
Proteose Peptone	98 ± 1	106 ± 8
C parvum	93 ± 6	96 ± 4
Chrysotile	96 ± 2	94 ± 9

$$\% \text{ cells phagocytic} = \frac{\text{no. cells containing more than 3 beads}}{\text{total no cells}} \times 100 \pm SD$$

$$\% \text{ viability} = \frac{\text{no. live cells per treated plate}}{\text{total no of cells per untreated plate}} \times 100 \pm SD$$

Results are a mean of 3 cultures.

Figure 6.7 The Morphological Appearance of Proteose Peptone-Elicited Macrophages 24 Hrs Following Exposure to UICC Crocidolite (100 µg). (x450).

Figure 6.8 The Morphological Appearance of Proteose Peptone-Elicited Macrophages 24 Hrs Following Exposure to UICC Chrysotile (100 µg). (x450).

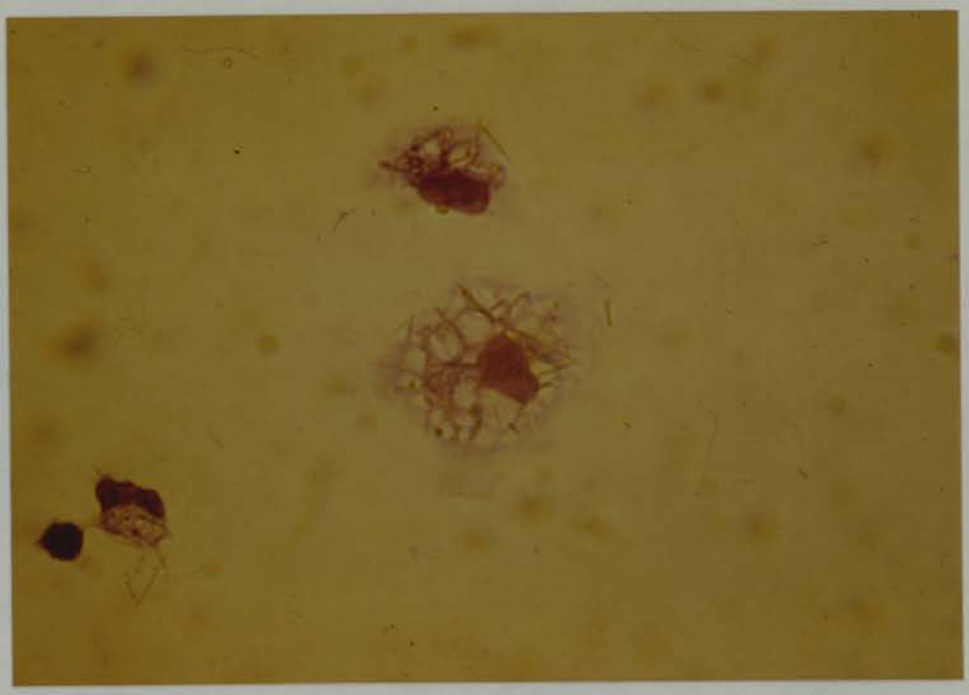

Figure 6.9 The Morphological Appearance of Crocidolite-Induced Macrophages 24 Hrs Following Exposure to UICC Crocidolite (100 µg). (x450).

Figure 6.10 The Morphological Appearance of Crocidolite-Induced Macrophages 24 Hrs Following Exposure to UICC Chrysotile (100 µg). (x450).

6.3.5 The Viability of Macrophage Populations 24 and 48 Hours Following Treatment With Asbestos.

The viability of each population of cultured macrophages was assessed 24 and 48 hrs following exposure to UICC crocidolite, amosite or chrysotile (100 μg/plate) (Figures 6.11 and 6.12). Treatment of each population of cells for 24 hrs with either UICC crocidolite or amosite (Figure 6.11) did not result in a significant decrease in the viability of the macrophages. Treatment with UICC chrysotile also had a negligible effect on the viability of either the unstimulated or saline-induced populations; the proteose peptone, C parvum, and latex-induced cells showed an enhanced susceptibility to the cytotoxic action of chrysotile compared to either the crocidolite or amosite treatment ($p<0.05$ between percentage viability values of amosite and chrysotile treated populations of proteose peptone, C parvum and latex-induced macrophages). The proteose peptone, C parvum and latex-induced populations of cells proved to be more susceptible to the cytotoxic action of chrysotile than the saline-induced macrophages ($p<0.05$ between percentage viability values of proteose peptone, C parvum, latex and saline-induced populations following treatment with chrysotile). Those populations of macrophages induced following injection with UICC crocidolite, amosite or chrysotile, however, possessed the greatest degree of susceptibility to the cytotoxic action of chrysotile ($p<0.01$ between viability of crocidolite, amosite or chrysotile-induced macrophages 24 hrs following treatment with chrysotile, and the remaining populations of cells following the same treatment).

After 48 hrs of exposure to asbestos (Figure 6.12) similar results were obtained to those observed at the 24 hrs time point. It should be noted, however, that the standard deviations around the mean values for the viabilities of the asbestos-induced populations had increased considerably, thereby suggesting an increased debilitation of these cells. In addition, the proteose peptone-induced cells showed an enhanced susceptibility to chrysotile.

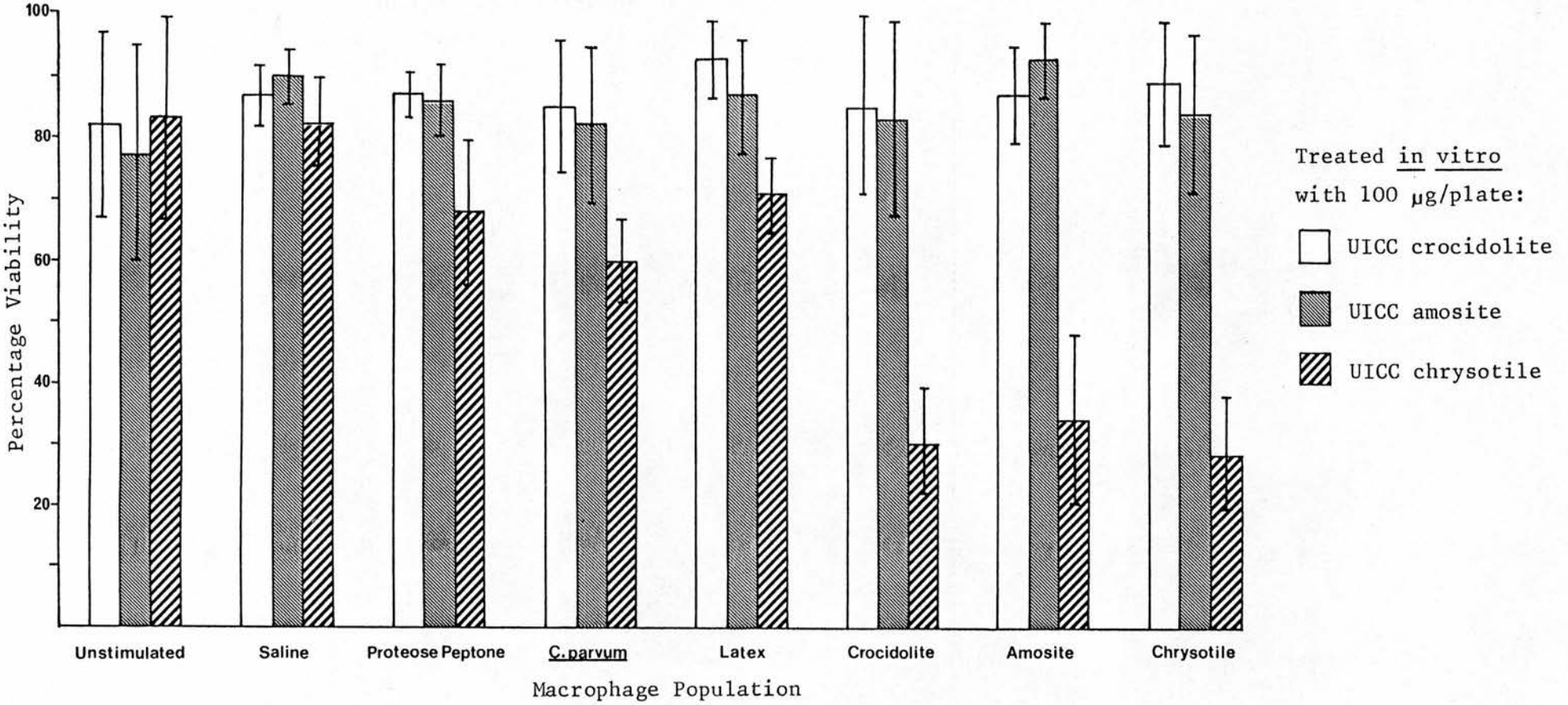

Figure 6.11 Percentage Viability of Macrophage Populations Following Exposure to Asbestos for 24 Hours.

Each value is a mean of at least 3 experiments ± SD.

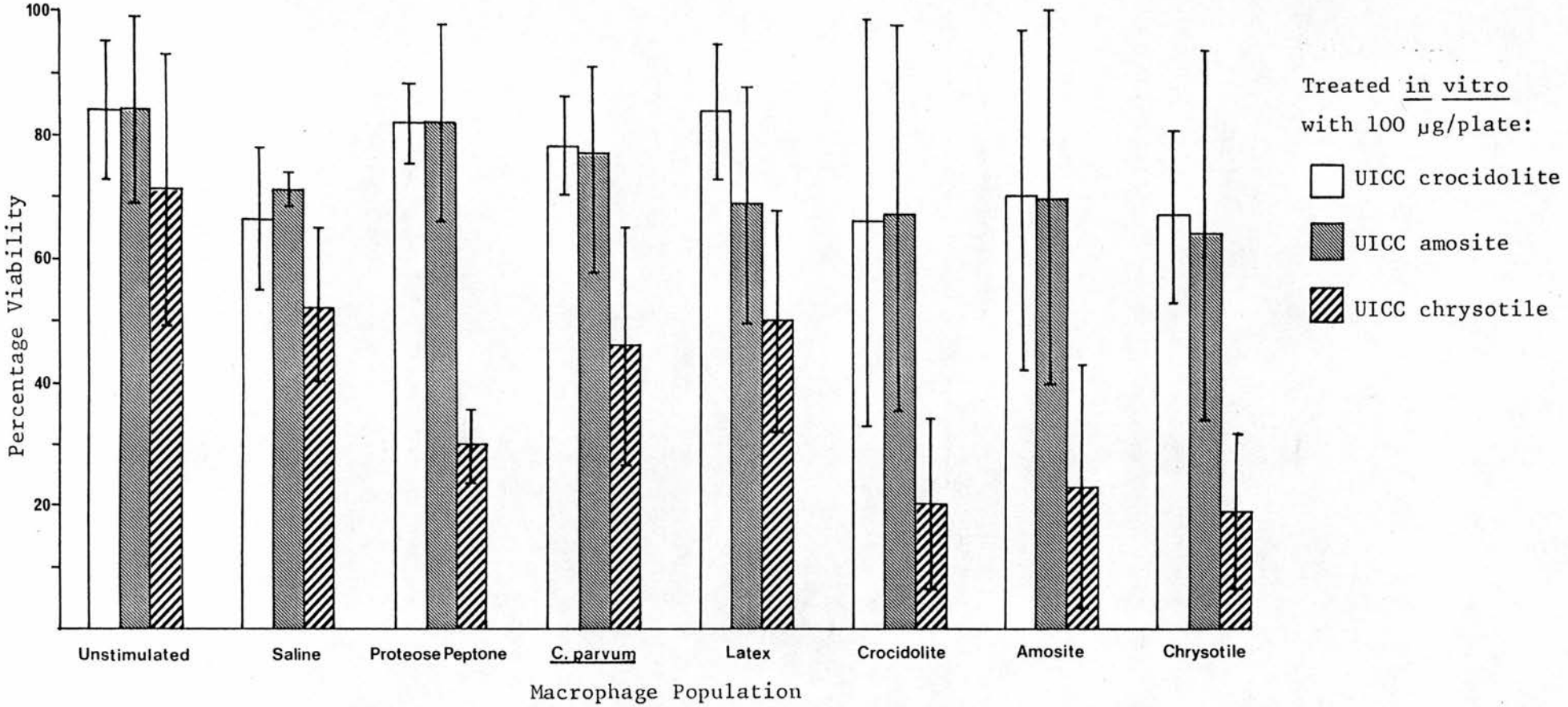

Figure 6.12 Percentage Viability of Macrophage Populations Following Exposure to Asbestos for 48 Hours.

Each value is a mean of at least 3 experiments ± SD.

6.3.6 The Viability of Lymphokine-Activated Macrophages 24 Hours Following Treatment With Chrysotile.

The viability of populations of macrophages (previously activated *in vitro* by exposure to lymphokine) following treatment with UICC chrysotile was assessed (Table 6.2). No significant differences were observed between the viability values obtained for the saline-induced control macrophages, lymphokine-activated and Con A treated macrophages following their exposure to UICC chrysotile.

6.3.7 Enzyme Release From Macrophage Populations Following Exposure to Asbestos.

Unstimulated, saline, *C parvum*, UICC crocidolite, amosite and chrysotile-induced macrophage populations were either untreated or exposed to 100 μg of UICC crocidolite, amosite or chrysotile. 24 hrs following treatment the overall percentage of LDH and glucosaminidase released into the medium was assessed (Figures 6.13 and 6.14). In general, the unstimulated, saline and *C parvum*-induced populations of macrophages released a similar quantity of LDH (approximately 10 to 20% of the total level per plate) into the medium regardless of the *in vitro* treatment. The asbestos-induced populations of cells proved more variable, however, and treatment with UICC chrysotile resulted in the release of a quantity of intracellular LDH which was significantly greater than the background observed for the control cells ($p<0.05$ between LDH release for control and chrysotile treated asbestos-induced populations of cells). The exposure of the unstimulated and saline-induced populations of macrophages to UICC chrysotile resulted in a release of glucosaminidase which was significantly greater than the levels released following treatment with either crocidolite or amosite ($p<0.05$). The treatment of *C parvum*, and asbestos-induced populations with UICC chrysotile involved the release of a quantity of intracellular glucosaminidase which was similar in value to the level released by unstimulated and saline-induced populations. In general, the glucosaminidase released by the asbestos-activated cells following chrysotile treatment did not differ significantly from the glucosaminidase release induced by treatment with crocidolite or amosite, and this was due to the large standard deviation around the means for these values.

Table 6.2 Viability of Lymphokine-Activated Macrophage Populations After 24 Hrs of Exposure to Chrysotile.

Macrophage population	% Viability after 24 hrs of exposure to chrysotile
Saline-induced macrophage	67.2 ± 7.0
Saline-induced macrophage + lymphokine	69.8 ± 9.2
Saline-induced macrophage + Concanavalin A	69.5 ± 8.7

$$\% \text{ viability} = \frac{\text{no. live cells following chrysotile exposure}}{\text{no. live cells in untreated control for each population of cells}} \times 100$$

Results are a mean of 3 experiments ± SD.

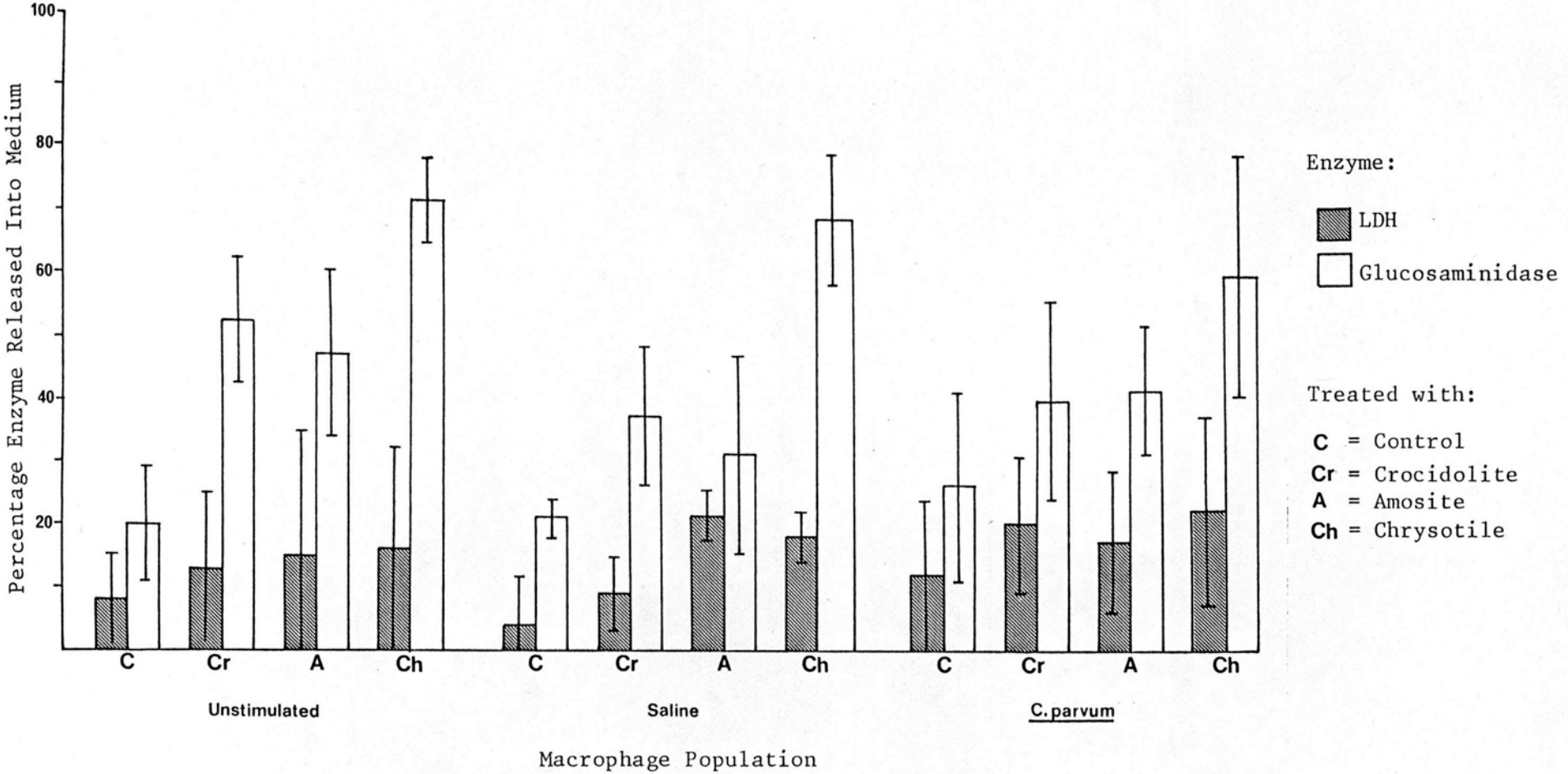

Figure 6.13 Enzyme Release Into Medium Following Exposure to Asbestos In Vitro for 24 Hours.

Each value is a mean of 3 experiments ± SD.

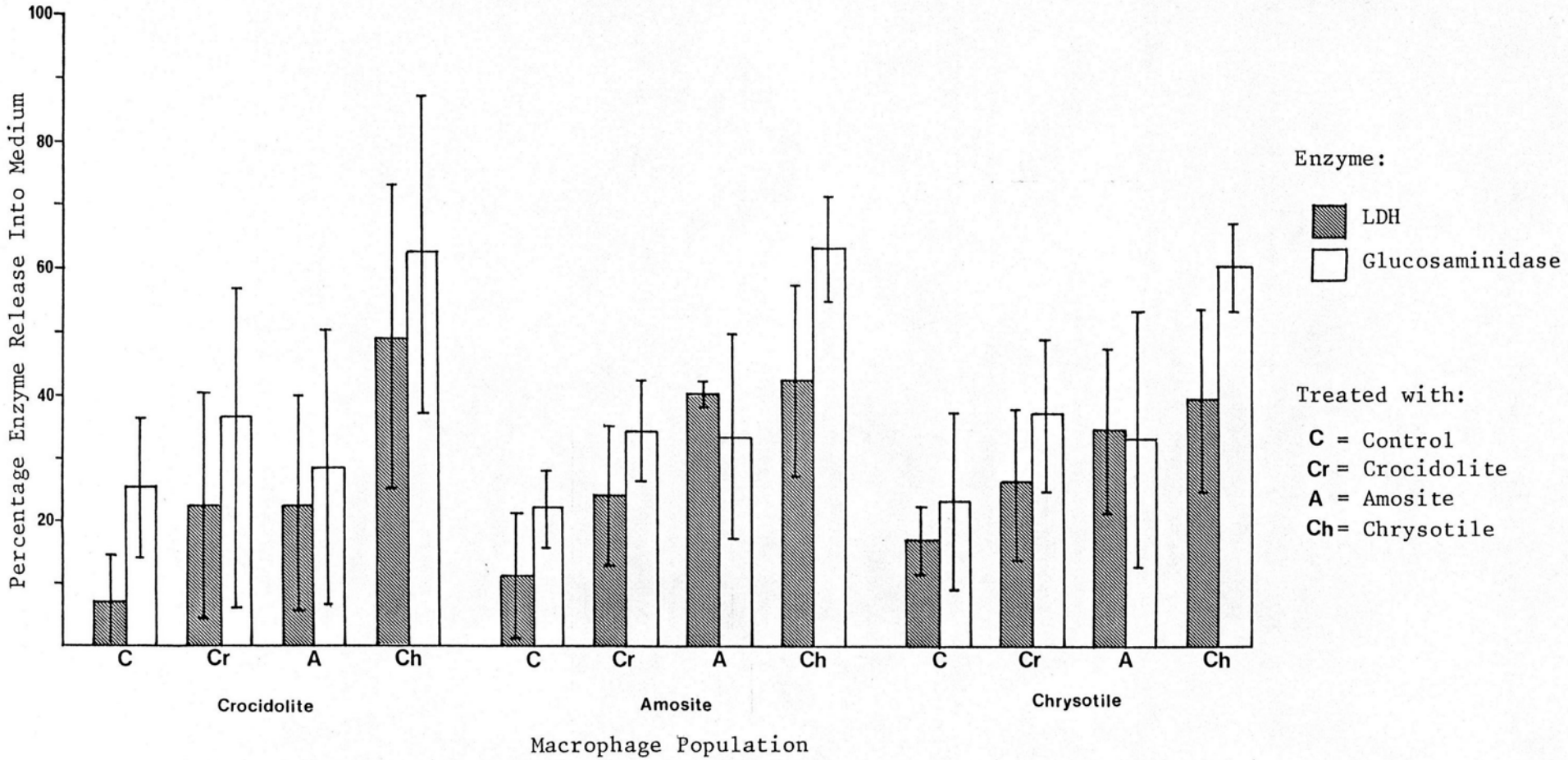

Figure 6.14 Enzyme Release Into Medium Following Exposure to Asbestos In Vitro for 24 Hours.

Each value is a mean of 3 experiments ± SD.

The exposure of the unstimulated and saline-induced cells to chrysotile resulted in a glucosaminidase release which was significantly greater than the LDH release observed ($p<0.05$); however, no significant difference between glucosaminidase and LDH release was noted following exposure of the C parvum and asbestos-induced populations of cells to UICC chrysotile.

6.3.8 The Electrical Properties of the Macrophage Membrane.

In order to assess the integrity of those populations of macrophages elicited by various agents in vivo the electrical properties of the cells were examined. The MP and IR values for each populations of cells is shown in Table 6.3. No significant difference for either the MP or IR values was observed between any of the populations of cells. An occasional spontaneous hyperpolarisation which achieved an MP value of -20 to -30 mV occurred within each population of macrophages.

Table 6.3 Membrane Electrical Properties for the Various Macrophage Populations.

Macrophage population	Membrane potential (mV)	Membrane resistance (MΩ)
Unstimulated	-10.6 ± 2.5	8.2 ± 5.3
Saline	-9.7 ± 1.8	9.2 ± 6.0
Proteose peptone	-10.9 ± 2.4	7.4 ± 3.3
C parvum	-10.2 ± 1.0	8.6 ± 3.8
Crocidolite	-9.6 ± 1.0	10.0 ± 4.0
Amosite	-9.3 ± 2.1	12.7 ± 7.7
Chrysotile	-9.7 ± 1.3	13.9 ± 8.0

Each value is a mean of at least 10 recordings ± SD.

6.4 DISCUSSION.

The ability of the macrophage to alter dramatically in both morphological appearance and functional activity, depending upon its activational status, has been recognised for a number of years (reviewed by Hopper et al, 1979). The possibility that macrophages may vary in their response to treatment with asbestos fibres, according to their state of activation has recently attracted some attention (Miller, 1978; Morgan and Allison, 1980). This study has therefore been designed to test the hypothesis that the susceptibility of a macrophage to the cytotoxic activity of asbestos fibres may differ according to its activational status. The mouse peritoneal cavity was used as a source of macrophages, and a variety of different agents, including UICC asbestos, were used to induce populations of stimulated and activated macrophages in vivo. All of these populations of elicited macrophages proved to contain cells at least 95% of which were viable (Section 6.3.1). Morphologically, the populations of cells were diverse, and those macrophages activated by exposure to the bacterial activating agent C parvum (Figure 6.3) had spread to a greater extent on the surface of the culture vessel and were more vacuolated than the unstimulated cells (Figure 6.1); a similar morphological alteration had also been noted by Wing and Remington (1978) following the in vivo exposure of macrophages to micro-organisms. Donaldson et al (1982) observed that asbestos had the ability to induce populations of macrophages that were similar in appearance to the C parvum-activated cells, and the results from this study confirm this finding (Figure 6.4 and 6.5).

In order to quantify the activational status of each macrophage population, a cell "spreading assay" was utilised (Donaldson et al, 1982) which comprised the assessment of the proportion of cells which showed the ability to spread completely onto glass within one hour of incubation. This assay proved to be convenient to use in the study, and had previously been shown to correlate well with such activational parameters as Fc receptor avidity (Donaldson et al, 1982). However, it must be borne in mind when interpreting this data that the spreading assay demonstrates only one activational characteristic of the macrophage, and may not necessarily denote the complete activational status of the cell. The unstimulated and saline-induced populations of macrophages

contained few activated cells, whereas the C parvum elicited populations of macrophages contained a high proportion of activated cells, and this proportion was similar to those observed for each of the asbestos-elicited populations (Figure 6.6), a finding that was also noted by Donaldson et al (1982). Intraperitoneal injection of latex beads did not result in the production of an activated population of macrophages; this result was in agreement with the finding of Mørland and Kaplan (1977), and illustrates that the exposure of macrophages to inert particles in vivo does not necessarily result in the activation of these cells.

In order to assess the phagocytic capacity of the PECs, each population of cells was exposed to latex beads. After 24 hrs all of the populations of cells displayed a similar phagocytic capacity (Table 6.1). Pesanti and Nugent (1981) observed that the phagocytosis of particulate material by macrophages in vitro can result in the inhibition of further phagocytosis during a second challenge with with particles; in contradiction of this theory, those populations of macrophages treated in vivo with latex or asbestos proved to be phagocytic. This assay was, however, only designed to illustrate the number of cells that were capable of phagocytosis, and it is therefore possible that a further examination of the rate of phagocytosis or even the overall number of particles phagocytosed may demonstrate a suppression of one or both of these facets of the phagocytic process.

The viability of each macrophage population was assessed following exposure to either latex beads, UICC crocidolite, amosite or chrysotile. The phagocytosis of latex did not result in a significant reduction of viability (Figure 6.1), thereby illustrating that the phagocytic process did not induce cell death, and this finding was also reported by McGee and Myrvik (1979). The exposure of each macrophage population to UICC crocidolite or amosite resulted in a high cellular viability (Figure 6.11 and 6.12), although an increase in the standard deviations around the mean values for the asbestos-induced populations at the 48 hrs time point was noted, which may have been due to an increased debilitation of these cells. The exposure of the macrophage populations to chrysotile, however, demonstrated that each population of macrophages possessed a different susceptibility to this fibrous sample, and in

particular those populations of cells that had received a prior exposure to asbestos *in vivo* displayed the greatest degree of susceptiblity *in vitro*. An attempt was made to enhance the susceptibility of macrophages to chrysotile following their pre-activation *in vitro* by lymphokine. The pre-activation process was performed according to the method of Lazdins *et al* (1978); this experiment was not successful however, as the "activated" cells showed a similar degree of susceptibility to chrysotile as the non-activated cells (Table 6.2). This situation was not examined further, but it is possible that a re-appraisal of the lymphokine-activating procedure would prove useful; for example the exposure of the macrophages to lymphokine for a longer period of time ie 48 or 72 hrs rather than 24 hrs, or alternatively the regular addition of fresh lymphokine might prove more successful.

The cytoplasmic and lysosomal enzyme release patterns observed for each population of cells following their exposure to UICC asbestos are shown in Figures 6.13 and 6.14. The pattern of LDH release upon treatment with asbestos proved to be similar for all populations of cells, with the exception of the asbestos-induced populations of cells which became more permeable upon the addition of chrysotile, although the large standard deviations around the mean figures for these values negated the statistical significance of this effect. The enhanced LDH release from the asbestos-induced populations of cells following exposure to chrysotile is in agreement with the data regarding the cellular viability. The membranes of the *C parvum*-activated cells following similar treatment does not agree with the apparent reduction in cellular viability. The membranes of the *C parvum*-activated cells may have become damaged by chrysotile in a manner that allowed the entry of the trypan blue molecules but not a loss of LDH; bearing in mind that the trypan blue molecule is smaller than the LDH molecule, the molecular weight being 960.8 rather than 140,000 (Merck Index, 1968; Sober, 1968), this would seem to be a reasonable explanation; the asbestos-induced cells may therefore have been damaged to an even greater extent. With regard to the release of the lysosomal enzyme glucosaminidase, the untreated cells from each population of macrophages released a similar background quantity of glucosaminidase during the initial 24 hr period of culture *in vitro*; this is in agreement

with the report of Hamilton (1980) who did not observe a difference in the release of lysosomal enzyme for either unstimulated or chrysotile-elicited macrophages. The pattern of glucosaminidase release following the exposure of all populations of cells to asbestos was in agreement with the previous reports in that chrysotile stimulated a greater release of lysosomal enzyme than amosite or crocidolite (Miller and Harington, 1972; Kaw and Zaidi, 1975; Davies, 1980b), regardless of the activational status of the cells. In summary, therefore, it would appear that chrysotile had the ability to induce a release of glucosaminidase from macrophages which was similar in quantity, regardless of the activational state of the cells; in addition the release was always greater than that induced by amosite or crocidolite. The chrysotile induced release of intracellular glucosaminidase was only accompanied by a loss of cytoplasmic LDH following treatment of the asbestos-induced populations of cells and could therefore be termed a cytotoxic effect. Chrysotile did, however, show the ability to induce a release of glucosaminidase in the absence of any significant loss of LDH from the unstimulated, saline or C parvum-induced populations of cells, and this could be termed a selective release of lysosomal enzyme. The results from the trypan blue assay suggest that the cell death induced by chrysotile was greater than would have been interpreted from the LDH assay; thus according to the trypan blue assay, chrysotile showed the ability to induce cell death in all populations of cells, with the exception of the unstimulated and saline-induced populations of cells. This suggests that the selective release of lysosomal enzyme noted by Davies et al (1974a) in the absence of cell membrane damage was associated with non-activated populations of cells only; and, more interestingly, even crocidolite and amosite showed the ability to induce a release of lysosomal glucosaminidase from the unstimulated populations of cells, although this was always lower than the level observed following chrysotile treatment. These observations were reported at the British Society for Cell Biologists meeting (Wright and Davis, 1981) and the Second International Workshop on The In Vitro Effects of Mineral Dusts (Wright et al, in press).

The reason why the activated, and in particular the asbestos-activated populations, should prove more sensitive to the cytotoxic action of chrysotile remains unclear. It has been suggested that the

process of macrophage activation requires an increase in the turnover of membrane phosphatidylinositol (Ögmundsdottir and Weir, 1980) thereby suggesting an increase in membrane fluidity which may be associated with an increase in membrane permeability. However, an examination of the electrophysiological properties of each population of macrophages showed that there was no difference between the MP and IR values for each type of cell, regardless of the activational state (Table 6.3). Previous reports have shown that the MP value for the macrophage increases in value upon stimulation with cellular activating agents such as chemotactic factors (Gallin et al, 1975) or lymphokine (Niemtzow et al, 1979). This increase in MP is possibly a manifestation of the early events involved in the macrophage activating process, whereas the results obtained in this study suggest that the MP and IR values subside to a constant value 24 hrs following culture in vitro, and in particular, the results indicate that the activated populations of macrophages do not possess cell membranes that are more permeable than the non-activated populations of cells.

Recent reports by McGee and Myrvik (1979) and McGee and Hale (1981) have shown that activated macrophages are more readily injured upon phagocytosis of toxic agents than non-activated cells, and this results from the release of a number of toxic by-products during the process of oxygen metabolism eg superoxide anion and hydrogen peroxide which are known for their membrane damaging activity (McCord and Wong, 1979). Activated macrophages can produce a greater quantity of these by-products (Bryant et al, 1982), and the release of a greater quantity of oxygen species following chrysotile ingestion by asbestos-elicited macrophages could initiate alterations in the cell membrane which would render these cells more susceptible to the cytotoxic action of chrysotile. A study concerning the release of such metabolites and their effect on the macrophage membrane during chrysotile ingestion would therefore be of some importance.

With regard to the loss of lysosomal enzymes, this study has provided additional material to support the previous observations that chrysotile can induce an enhanced release of lysosomal enzymes, but has not yet demonstrated the reason for this occurrence. Early

theories concerning the selective release of lysosomal enzymes following interaction with inflammatory agents proposed that these agents, which are also activators of the alternative pathway, induce the macrophage to release C3b which in turn stimulates lysosomal enzyme release (Schorlemmer et al, 1977a). Schorlemmer et al (1977b) also reported that unstimulated macrophages do not release C3b, but the present study has shown that chrysotile, a known activator of the alternative complement pathway (Saint-Remy and Cole, 1980) is capable of inducing the release of similar quantities of glucosaminidase from all types of macrophage, regardless of their activational state. This would suggest that chrysotile may elicit the enzyme release by a mechanism which is different to the one previously described. Riches and Stanworth (1982) have recently provided evidence to support their theory that acid hydrolase secretion can be initiated by increasing the localised pH of the lysosome, and this release is completely independent of complement activation. It is known that chrysotile loses the surface magnesium hydroxide layer when leached in physiological solution, and this can create a pH of approximately 10 (Chowdhury, 1973). It is possible, therefore, that ingested chrysotile may alter the pH of the lysosome, thereby initiating a selective release of lysosomal enzyme. This theory is supported by the finding of Jaurand et al (1980a) who reported that acid-leaching the surface of chrysotile reduced its ability to initiate selective release. A further analysis of the ability of various modified dusts to elicit acid hydrolase release, with reference to their abilities to affect lysosomal pH, may therefore prove useful.

This study has shown that the response of a population of macrophages to treatment with a cytotoxic dust in vitro may vary, and is dependent upon the activational status of the constituent cells. The reason for the enhanced sensitivity of the activated macrophages has not been provided and it is hoped that further research into this problem will clarify this situation. It is of interest to note that those populations of cells which had received prior exposure to asbestos in vivo should prove to be particularly sensitive to the cytotoxic action of chrysotile in vitro. It would be expected that an "occupationally exposed" human alveolar macrophage would receive several challenges

of asbestos dust or other activating agents, and should the alveolar macrophage respond in a similar manner to the peritoneal macrophage, the results from this study would indicate that these cells may be particularly susceptible to the cytotoxic action of further inhaled particles.

6.5 CONCLUSIONS.

This study comprised an examination of the response of populations of macrophages, induced by different stimulating and activating agents *in vivo*, to treatment with UICC asbestos *in vitro*. UICC crocidolite and amosite proved to be non-cytotoxic towards all populations of cells; the susceptibility of each population to chrysotile varied, and the more activated macrophages, in particular the asbestos-activated cells, showed a high degree of susceptibility to the cytotoxic action of chrysotile.

CHAPTER 7 GENERAL DISCUSSION AND CONCLUSIONS.

Asbestos fibres are well known for their ability to induce a number of diseases in man upon gaining access to the internal milieu of the host. These disease states include asbestosis, bronchogenic carcinoma and pleural and peritoneal mesothelioma (Becklake, 1976), and all three conditions have been associated with the main commercially available asbestos types (Selikoff and Lee, 1978). An examination of the main asbestos types has shown that they differ considerably in ionic content and structure (reviewed in Sections 1.2 and 1.3), and considerable research effort has therefore been directed towards identifying the pathogenic factor that is common to all of the asbestiform minerals. A number of limited epidemiological studies have shown that the longer fibres may be more pathogenic than the shorter ones (Timbrell et al, 1971; Becklake, 1976), and this possibility has been substantiated by data from animal studies (reviewed in Section 1.12.2) and also in vitro studies involving macrophages and permanent cell lines (reviewed in Sections 1.13.5.4 and 1.13.6).

The mechanism by which asbestos fibres initiate disease in man has been extensively studied for a number of years, in particular the effect of these fibres on potential target cells in the body has warranted attention (reviewed by Harington et al, 1975; Selikoff and Lee, 1978). The alveolar macrophage is considered to be one of the first cells in the body to interact with the asbestos fibres following their inhalation from the atmosphere (Allison, 1968); this cell is actively involved in the phagocytosis and removal of inhaled particles from the lung, as well as playing several other important roles in the body, including immunological mediation, the production of a variety of secretory products, and the destruction of microbes and tumour cells (reviewed in Section 1.14). It is evident, therefore, that the study of the effects of asbestos fibres on macrophages is of importance, in particular with regard to the ability of the fibres to modify the normal functioning of the cells thereby resulting in the exposure of the host to a variety of modified secretory products accompanied by an alteration in immune status and possible enhanced susceptibility to disease.

The study of the direct effect of asbestos fibres on this particular cell type has therefore commanded considerable attention, in particular with regard to the ability of each asbestos type to modify the normal functioning of the macrophage in vitro. Early studies showed that the amphiboles had little effect on macrophages, whereas the serpentines showed the ability to cause cell death (reviewed by Harington et al, 1975), and in some instances chrysotile could also induce a selective release of lysosomal hydrolases in vitro (Davies et al, 1974a). The possibility that asbestos could induce a release of inflammatory agents either via selective release or cell death awakened considerable interest with regard to the tentative usefulness of the macrophage for incorporation in a predictive assay system (reviewed by Harington et al, 1975; Harington, 1976; Davies and Allison, 1976). The examination of the macrophage/asbestos fibres interaction continued; later studies provided evidence to support the hypothesis that longer fibres are more cytotoxic towards macrophages (Davies, 1980a;Davies et al, 1980; Kaw et al. 1982), and Chamberlain et al (1979) demonstrated a close correlation between enzyme release and the number of fibres greater than 10 μm in length. Wade et al (1980) examined the effect of defined fibrous samples on $P388D_1$ macrophage-like cells and showed a close association between the cytotoxic capacity and the number of fibres longer than 8 μm in each sample. In addition, Wade et al (1980) also demonstrated that the results from the $P388D_1$ cell assay were in agreement with the results obtained from in vivo tumour induction studies. Further evidence was required, however, to corroborate the existence of a length threshold above which fibres may become pathogenic towards macrophages and also to ascertain the usefulness of the results obtained from the study of this cell in predicting the in vivo pathogenicity of fibrous samples.

This study was designed to examine further the effects of asbestos fibres on macrophages, in particular with regard to asbestos-induced alterations in membrane permeability and enzyme release. It has been shown that macrophage populations may vary depending upon their activational status (Hopper et al, 1979), and it was suggested that each population may therefore vary in their response to treatment with asbestos (Miller, 1978; Morgan and Allison, 1980); in order to overcome

this problem the $P388D_1$ macrophage-like cell line was used in this study. Previous reports had suggested that a close relationship may exist between the cytotoxic potential of a dust sample, its fibre length content and also the *in vivo* pathogenicity (Davies, 1980b; Brown *et al*, 1980; Wade *et al*,1980); The relationship between fibre length and *in vitro* cytotoxicity was therefore examined in order to provide information which would be useful for future comparisons with *in vivo* data, thereby determining the predictive nature of this *in vitro* assay. As it has been suggested that the response of a macrophage to treatment with asbestos may vary depending upon the nature of prior stimulating agents (Miller, 1978; Morgan and Allison, 1980), the alterations in membrane permeability and enzyme release by macrophages in different activational states following their exposure to asbestos was established, as this may reflect the occurrence of differences in their response *in vivo*.

A number of asbestiform samples were made available for this study, these included a selection of amphiboles and serpentines from different sources as well as samples of chrysotile prepared by the industrial wet dispersion process (Heron and Huggett, 1971); these have been described in Section 2.2.2. All samples were passed through an "elutriation" process so that each type of mineral contained only the respirable portion of fibres, thereby ensuring that the *in vitro* experiments comprised the examination of those fibres to which the body's cells would be exposed *in vivo*. Bearing in mind that one of the objectives of this study was to establish the relationship between fibre length and *in vitro* cytotoxicity, it proved necessary to assess the fibre length distribution and fibre number content of each dust sample. The techniques available for assessing the fibre number involved the use of either the light microscope, SEM or TEM. The light microscope was discounted because of its poor resolution (Ruud, 1978; Le Guen *et al*, 1980); the TEM was also excluded because of the possibility of the occurrence of fibre loss or breakage during the preparation procedure, in addition the use of the TEM grid support was also excluded because of the problems associated with the measurement of those long fibres that may traverse the grid bars. The SEM was therefore the obvious instrument to use, and the preparation techniques associated with its use proved to be simple and highly reproducible (Section 2.3.2.1).

Nuclepore filters were utilised to support the fibres for SEM observation; a preliminary examination of the 0.4 μm pore filters gave results which suggested that a proportion of the finer fibres had been lost, and the adoption of the use of the 0.2 μm pore filter improved this situation (Section 2.4.1). It did not prove possible to assess the fibre number content and fibre length distributions of those samples prepared by the wet dispersion process because of the inability of the author to disperse the individual fibres adequately in solution. There were no problems associated with the assessment of the remainder of the asbestos samples, and the results are described in Section 2.3.3.3 and 2.3.3.4. Unfortunately there were only a few publications available with which the data from this study could be compared, and the authors of these publications had used the TEM in their studies (Timbrell, 1969; Langer et al, 1974; Coffin and Palekar, 1978; Brown et al, 1978; Rendall, 1980). The fibre number and fibre length distributions obtained in this study were similar to those noted by Brown et al (1978), however, some differences were observed between the remaining publications (Timbrell, 1969; Langer et al, 1974; Coffin and Palekar, 1978; Rendall, 1980) and the present study, and these were attributed to differences in preparation technique. In general, a greater proportion of finer fibres were observed using the TEM (Langer et al, 1974; Rendall, 1980), and this was probably because of the superior resolving power of the instrument. It could be concluded therefore, that whilst the preparation and counting techniques associated with the use of the SEM were highly reproducible, TEM observation would more accurately detect the finer fibres within each population. It is probable, however, that the use of the newer SEM models, which have a better resolution, would give results that may be similar to or even improve upon those obtained from TEM studies.

A major objective of this study was to examine the mechanism of interaction of asbestos fibres with the macrophage. As a number of reports had comprised descriptions of the successful use of the erythrocyte membrane for the study of membrane/particle interactions (Allison, 1971; Gabor and Anca, 1974; Depasse, 1977, 1980; Light and Wei, 1980), a preliminary examination of this simple model was carried out to establish whether the results would aid in the interpretation of data from the macrophage experiments (Chapter 3). A number of

fibrous samples were assessed according to their ability to lyse sheep erythrocytes, and similar results were obtained to those reported previously in that the serpentine samples were more haemolytic than the amphiboles (Harington et al, 1971b; Light and Wei, 1980). This test system did not prove useful for the examination of the E WDC sample, however, because of the inability of the author to disperse the fibres adequately in suspension; this result illustrated the requirement for adequate fibre/cell contact in order that the system should function correctly. More recently the haemolysis assay has fallen into disrepute as a number of research groups, whilst finding a general correlation between the haemolytic potential of a dust sample and its cytotoxic activity towards macrophages, have also observed a few discrepancies (Daniel and Le Bouffant, 1980; Gormley et al, 1980; Wright et al, 1980); and Richards et al (1980) did not find a relationship between the haemolytic capacity and in vivo pathogenicity of his dust samples. The haemolysis assay was not examined further in this study, and whilst the erythrocyte membrane may be a useful model for the examination of fibre/membrane interactions it must be considered that the results from this assay may not necessarily reflect the mechanism of interaction of fibres with the macrophage membrane.

Before examining the cytotoxic effect of various asbestos samples on macrophages, the choice of the macrophage population to be studied was considered; the morphological, biochemical and functional characteristics of a macrophage population may vary considerably depending upon the source and also the nature of any prior stimulating agents to which the cells have been exposed (Hopper et al, 1979). In order to avoid this choice and also to reduce the loss of animal life that would be associated with the use of primary macrophages, a permanent cell line was chosen (Section 4.14). The $P388D_1$ cell line (Dawe and Potter, 1957) was examined because of its many macrophage-like characteristics (Section 4.1.3),and successful use in previous cytotoxicity assays (Wade et al, 1976; Gormley et al, 1979); although it was constantly borne in mind that these cells were not macrophages and therefore may not adequately reflect the activity of all types of macrophage. The $P388D_1$ cells were exposed to a variety of different types of asbestiform fibres at dust concentrations of 10 and 50 μg/ml of medium.

After 24 and 48 hrs of exposure a number of "cytotoxicity tests" were carried out: i) the ability of the cells to exclude trypan blue to establish membrane damage, ii) LDH release to establish membrane damage, iii) glucosaminidase release to establish the existence of "selective release" or cell damage, iv) lactate production to establish an alteration in the metabolic activity of the cells. In general, the order of cytotoxic potential of the dusts, according to the trypan blue exclusion test, was in agreement with previously reported data, in that the serpentines were more cytotoxic than the amphiboles (Harington et al, 1975; Wade et al, 1976) (Table 4.5); anthophyllite and tremolite were more cytotoxic than crocidolite (Davis, 1980b; Robock and Klosterkötter, 1973), and heated chrysotile was more cytotoxic than the original parent sample (Hayashi et al, 1969). The most cytotoxic of the asbestiform minerals proved to be the industrially prepared WDC samples (Table 4.15), and this high cytotoxic capacity was shown to be due to the integral structure of the fibres themselves rather than the presence of any contaminating components acquired during the preparation procedure (Section 4.3.4.1 and 4.4.1).

A comparison between the respective cytotoxic potentials of the dust samples, according to their abilities to alter the various measured cytotoxicity parameters, showed that a more cytotoxic sample had a greater ability to damage the cell membrane thereby allowing the entry of trypan blue (Table 4.5 and 4.15) and the release of intracellular LDH (Table 4.7 and 4.17), in addition an increase in the release of lysosomal glucosaminidase was observed (Tables 4.11 and 4.21). A significant positive correlation was noted for the relationship between the reduction in viability, intracellular LDH and glucosaminidase following treatment with 10 or 50 μg/ml of fibres for 24 or 48 hrs ($p<0.001$) (Figures 4.26 and 4.29). The measurement of the lactate production following dust treatment did not prove useful, however, as significant alterations in lactate output were only observed following treatment of the cells with the more cytotoxic WDC samples (Tables 4.6 and 4.16). This particular assay has been previously used successfully by Beck et al (1971), but was not adequately sensitive to detect the low levels of cell damage induced by the less cytotoxic dusts in the $P388D_1$ system (discussed in Section 4.4.2). A previous report by

(Davies et al, 1974a) had shown that chrysotile could elicit a selective release of lysosomal enzymes in the absence of any loss of LDH; no evidence however, for the existence of this type of release following chrysotile ingestion was observed in the $P388D_1$ system. The interpretation of the values obtained for the levels of glucosaminidase in the medium proved confusing because of the apparent decay of the activity of this enzyme upon its release into the medium (discussed in Section 4.4.2). This finding, when considered together with the lack of "selective release", casts some doubt upon the usefulness of the continued utilisation of this particular assay; certainly the relationship between the glucosaminidase release and the remaining assays was adequately significant ($p<0.001$) to suggest that the measurement of one assay alone would be sufficient to establish the occurrence of cell damage. The measurement of either LDH release or trypan blue exclusion to estimate the increase in membrane damage would perhaps be the most suitable for routine use.

One of the most sensitive assays available for assessing the integrity of the cell membrane is the measurement of the electrophysiological properties ie the examination of any alteration in the permeability of the membrane and also resistance to ionic flow. The assessment of both MP and IR values had been successfully used previously to demonstrate the occurrence of membrane perturbations following the treatment of macrophages or $P388D_1$ cells with dust (Gormley et al, 1978; Wright and Gormley, 1980; Gormley and Wright, 1980), and this technique was therefore examined in this study (Chapter 5) in order to establish its usefulness for routine use. An examination of the effect of the UICC asbestos samples on the electrophysiological characteristics of the $P388D_1$ cell membrane (Section 5.3.2) showed that the more cytotoxic serpentine sample caused an earlier decline in both MP and IR values than the amphiboles, thereby indicating that an increase in the permeability of the membrane to ionic flow had occurred. The UICC amosite and also a number of amosite samples from various sources showed the ability to induce a significant cell membrane hyperpolarisation ($p<0.05$) (Section 5.3.3) during the first hour of exposure to fibres. This finding was in agreement with the results obtained by Gormley and Wright (1980), and a similar response had also been noted following

the exposure of macrophages to chemotactic factors (Gallin and Gallin, 1977) and calcium ionophores (Oliveira-Castro and Dos Reis, 1981). An examination of this phenomenon showed that the amosite fibres were inducing the cell membrane hyperpolarisation by activating the Ca^{++}-sensitive K^{+} channel (Section 5.4.2). The question then arose as to the nature of the agent responsible for the induction of the hyperpolarisation. Morgan and Cralley (1973) observed that Mn^{++} contaminates UICC amosite at a level of 13000 ppm; the addition of Mn^{++}, in the form of $MnCl_2$, to $P388D_1$ cells also resulted in the induction of a cell membrane hyperpolarisation which was shown to be caused by activation of the Ca^{++}-sensitive K^{+} channel (Section 5.3.5 and 5.3.6). It is known that certain heavy metal ions can interfere with the macrophage membrane, thereby reducing the tumour cell cytotoxic capacity of these cells (Nelson et al, 1982). The possibility that an ionic contaminant available on the surface of asbestos fibres may have such a profound effect on the electrical activity of the cell membrane has interesting connotations; it is interesting to speculate that certain ionic components may interfere with the macrophage membrane thereby resulting in the suppression of certain functions of this cell. The electrophysiological assay proved particularly useful for the study of the mechanism of interaction of mineral fibres with the cell membrane; it could not be recommended for routine use, however, because of the considerable operator bias and the time involved in the examination of each cell. It must be concluded, therefore, that the assays most suited for routine use in a cytotoxicity system are either the measurement of LDH release or the ability of the cells to exlude trypan blue.

The results from the electrophysiological studies, whilst providing a useful insight into the mechanism of interaction of one type of fibre with the cell membrane, did not explain the reason for the variation in the cytotoxic abilities of the different types of fibre. It was a prime aim of this study to establish the role of fibre length in determining the cytotoxic capacity of a fibrous sample, and a comparison was made between the ability of 13 selected fibrous samples to modify the release of intracellular LDH and glucosaminidase, the ability of the cells to exclude trypan blue, and the constituent fibre length content of each sample (Section 4.3.6.2). In general,

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a close correlation was observed between the number of fibres greater than 8 μm in length and the three cytotoxicity parameters ($p<0.05$), and this was in agreement with the findings of Wade et al (1980) and Lipkin (1980) who examined the relationship between fibre length and alterations in the growth curves of $P388D_1$ cells. A few discrepancies were observed, however, and it was noted that the exposure of the cells for 24 hrs resulted in a loss of LDH that corresponded to the number of fibres greater than 20 μm for the dust concentration of 10 μg/ml, and 10 μm for the 50 μg/ml concentration. This result was ascribed to the low degree of sensitivity of the LDH assay to minimal levels of membrane damage (Section 4.4.3). Results from the glucosaminidase assay showed that fibres of greater than 8 μm in length caused the greatest release of glucosaminidase following the treatment of cells with a dust concentration of 50 μg/ml and this release could be linked to a cytotoxic effect. The treatment of the cells with the lower concentration of dust, however, showed that fibres of greater than 1 or 2 μm stimulated a release of lysosomal enzyme which was not necessarily linked to membrane damage. This finding was of interest because it had not been reported previously and was in contrast to a previous publication which linked the longer fibres to enzyme release (Davies, 1980b). In general, however, the finding that the longer fibres were more cytotoxic towards macrophage-like cells was in agreement with previous reports for both $P388D_1$ cells and macrophages (Wade et al, 1980; Chamberlain et al, 1979; Davies, 1980b; Kaw et al, 1982). The reason for the enhanced cytotoxic capacity of the longer fibres towards macrophages has not been shown in this study, although the theory of Johnson and Davies (1980) that these cells are not able to cope with long fibres and that disruption of the cytoskeletal components may occur, must be considered. One important criticism of the present study is that the dimensions of only 200 fibres were counted in accordance with the number normally routinely counted in a fibre sample at the IOM; only a small proportion of the 200 fibres were greater than 6 μm in length and this effectively reduces the statistical significance of the finding that cytotoxicity is related to number of fibres greater than 8 μm in length. 1000 fibres were counted in order to estimate the fibre number content of each sample, and this study would have been greatly improved if the dimensions of all of the 1000 fibres had also been measured.

The final portion of this study comprised an examination of the cytotoxic effect of UICC crocidolite, amosite and chrysotile towards macrophages in different activational states (Chapter 6). Populations of mouse peritoneal macrophages were induced in vivo with a variety of stimulating agents including asbestos, and each population was different in morphological appearance (Section 6.3.1) and activational status (Section 6.3.2). The populations proved to be equally phagocytic and no cell death was noted following treatment with inert latex beads. (Section 6.3.3). UICC crocidolite and amosite also proved to be non-cytotoxic towards all populations of cells. The response to treatment with chrysotile varied and the more activated cells, in particular the asbestos activated populations of cells, proved highly susceptible to the cytotoxic action of chrysotile (Section 6.3.5). With regard to the release of lysosomal and cytoplasmic enzymes (Section 6.3.7) it was noted that, in agreement with previous reports (Miller and Harington, 1972; Kaw and Zaidi, 1975; Davies, 1980b), chrysotile had the ability to induce a greater release of lysosomal enzymes from all populations of cells than did the amphiboles. The glucosaminidase release generally occurred in the absence of any significant loss of intracellular LDH, with the exception of the asbestos-induced populations in which an enhanced loss of LDH was observed. The loss of intracellular LDH did not always correspond to the ability of the macrophage membrane to exclude trypan blue, and this was again attributed to the greater sensitivity of the trypan blue assay. The reason for the enhanced susceptibility of the asbestos-induced macrophage populations is not evident, although measurements of the electrophysiological properties of the macrophage membrane showed that this was not due to an initially high permeability of these cells (Section 6.3.8). It is probable that the activated macrophages may produce a higher proportion of toxic oxygen species (Bryant et al, 1982) which may be released during phagocytosis thereby rendering the cell membrane more susceptible to the cytotoxic action of chrysotile. The reason for the enhanced release of lysosomal enzyme following chrysotile ingestion by all populations of macrophages also remains unclear, although recent evidence by Riches and Stanworth (1982) would indicate that selective release is initiated upon the increase of lysosomal pH. Chrysotile may create a pH of 10 upon leaching in solution (Chowdhury, 1973), and it must therefore be considered that

this alteration in pH may result in the observed enhanced release of lysosomal enzyme. It must be borne in mind that the response of the macrophage may also vary depending upon the nature of any agent coating the surface of the fibres, as a study by Davies (1980b) has shown that the pattern of enzyme release by macrophages may differ depending on whether the fibres are coated with serum or lung surfactant components. Only future research, however, will establish the accuracy of the aforementioned hypotheses and also the possible relevance of this study to the alveolar macrophage. It may be found that the response of the alveolar macrophage to treatment with dust can also vary in a similar fashion to the peritoneal macrophage. It is probable that the alveolar macrophage would become activated upon initial challenge with dust (Davis et al, 1980), and would display an enhanced susceptibility to the cytolytic action of a second inhaled challenge with dust, thereby releasing inflammatory agents and possibly contributing to enhanced fibrotic sequelae.

Another aim of this study was to establish the usefulness of the macrophage in vitro system, in particular the macrophage-like $P388D_1$ cell system for incorporation into an assay for the prediction of the in vivo pathogenicity of dust samples. The results obtained from this study would suggest that the $P388D_1$ system may indeed be useful, although further validation and comparison with data from animal experiments is required. There is considerable data from epidemiological, in vivo and in vitro studies to support the hypothesis that longer fibres are more pathogenic than shorter ones (reviewed in Sections 1.11.2, 1.12.2, 1.13.5.4 and 1.13.6)., and this work has provided further evidence to support this data. A comparison between in vitro and in vivo studies has already demonstrated that results from the $P388D_1$ system show a close agreement with results from animal tumour induction experiments (Wade et al, 1980), and, in agreement with the "Stanton Hypothesis", demonstrated that fibres longer than 8 μm in length are pathogenic. Two other cell systems which have been used to predict in vivo tumourigenicity are the A549 and V79-4 systems (Chamberlain et al, 1980); a comparison by Gormley et al (in press) has shown that results from the A549 and V79-4 and $P388D_1$ systems would indicate the same order of cytotoxic potential for a given series of dust samples.

Whilst this finding is encouraging, it will, however, be some time before the corresponding animal studies, with which this data can be compared, will be complete; it is therefore not possible to state at this time whether results from the $P388D_1$ system can accurately predict *in vivo* pathogenicity. The current structure of the $P388D_1$ system does not allow for a differentiation in the prediction of the fibrogenicity or tumourigenicity of fibrous samples but suggests only that the sample is pathogenic. Chamberlain and Brown have developed an *in vitro* assay system for the prediction of *in vivo* tumourigenicity based on the observation that tumourigenic fibres have the ability to induce multinucleate or giant cell formation in A549 cells (Chamberlain and Brown, 1978; Chamberlain *et al*, 1980). It was observed during an examination of the effects of dust samples on the morphology of $P388D_1$ cells, that fibrous samples alone could increase the occurrence of multinucleate cells (Section 4.2.6), and it is therefore possible that a comparison of the ability of a fibrous samples to stimulate multinucleate cell formation with its *in vivo* pathogenicity may result in the development of an assay system which can differentiate between the two disease forms. In this study the $P388D_1$ cell has proven to be a useful substitute for the primary macrophage; it was noted that these cells did not selectively release lysosomal enzymes upon chrysotile ingestion, but as previous work had suggested that the membrane of the activated macrophage may be more susceptible to the cytotoxic action of chrysotile, it is possible that the results from the $P388D_1$ cell reflect those that would be obtained from a highly activated macrophage.

7.1 CONCLUDING REMARKS.

This study was designed to examine in depth the cytotoxic interaction of asbestos fibres with macrophages *in vitro*, with particular regard to the release of potentially toxic agents caused by cell damage. In addition, the relevance of these studies to the *in vivo* situation with regard to the induction of fibrosis and cancer was considered. Data has been accumulating from *in vivo* studies to suggest that those fibres greater than 8 μm in length in each fibre population are the most carcinogenic (Stanton *et al*, 1977; Stanton and

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Layard, 1978). A later study by Wade *et al* (1980) showed that Stanton *et al*'s (1977) fibre samples had the ability to reduce the growth of macrophage-like $P388D_1$ cells, and demonstrated a close association between results from the *in vitro* $P388D_1$ cell assay and the *in vivo* tumourigenicity assay described by Stanton (Stanton *et al*, 1977; Stanton and Layard, 1978). The present study, which has also utilised $P388D_1$ cells has not only confirmed the report by Wade *et al* (1980) that the ability of a fibrous samples to reduce cell growth is dependent upon those fibres greater than 8 μm in length, but has also shown a close association between fibre length and the loss of intracellular hydrolytic enzymes. If hydrolytic enzyme release is linked to the induction of the inflammatory process (Davies and Allison, 1976), it may be considered that those fibres longer than 8 μm could possess a greater fibrogenic capacity *in vivo* than shorter fibres. Certainly there is data available from previous *in vivo* studies to indicate that longer fibres are more fibrogenic than shorter ones (reviewed by Harington *et al*, 1975); and a future comparison between the *in vivo* fibrogenicity of the fibrous samples used in this study and their ability to cause cell death and/or enzyme loss from $P388D_1$ cells *in vitro* should demonstrate the usefulness of the $P388D_1$ cell assay for the prediction of *in vivo* fibrogenicity.

Wade *et al* (1980) noted a close association between *in vivo* tumour induction by fibrous samples, the retardation of $P388D_1$ cell growth and the number of fibres longer than 8 μm. The present study has confirmed the association between fibres longer than 8 μm and loss of cellular viability, and this data should provide a sound basis for comparison with future studies investigating the ability of these samples to induce tumours *in vivo*. Future epidemiological studies will hopefully provide evidence to support the accumulating data from *in vivo* and *in vitro* studies demonstrating that longer fibres are more fibrogenic and carcinogenic than shorter ones.

Should future studies demonstrate that the $P388D_1$ cell can be successfully used for the prediction of fibre pathogenicity, it must be considered that the current structure of the $P388D_1$ cell assay system does not allow the distinction between fibrogenicity and

tumourigenicity. The A549 system, currently used for the prediction of tumourigenicity, was devised by Chamberlain and Brown (1978) and relies on the assessment of the induction of multinucleate giant cells; multinucleate cell formation also occurred in the $P388D_1$ cell cultures following their treatment with fibres but not other particulate dust samples (Section 4.2.6). A further study comparing multinucleate cell formation with tumour induction, and cell death with fibrogenicity may result in the development of an *in vitro* $P388D_1$ cell assay system which can be used to differentiate between the two disease states. Multinucleate cell formation by macrophages has been observed at a number of sites of the body during the generation of the inflammatory process (reviewed by Papadimitriou and Walters, 1979); the mechanism by which the formation of macrophage polykarya occurs has not yet been proven, and the $P388D_1$ cell may prove to be of use as a model permanent cell line for the study of this phenomenon.

The estimation of the fibre length distribution of each of the fibrous samples examined in this study has relied upon the use of an SEM model of limited resolving power. Whilst this study has shown that the preparation techniques and counting procedures were both technically simple and highly reproducible, it must be considered that a proportion of the smaller and finer fibres may not have been observed. It is evident, therefore, that a further study using a more modern SEM with a higher resolving power is necessary, and comparisons with results from TEM studies would be important in order to establish the superiority of the technique.

Recently, following the accumulating epidemiological evidence that asbestos can induce a number of immunological alterations in man (Rickards and Barrett, 1958; Lange *et al*, 1974; Kagan *et al*, 1977), a number of authors have examined the effects of asbestos on the immunological system *in vivo*. Miller *et al* (1980) and Donaldson *et al* (1982) have shown that asbestos has the ability to induce activated macrophages *in vivo*, although these cells do not possess the ability to kill tumour cells (Donaldson *et al*, 1982). The use of a technique which measured the electrophysiological properties of the cell membrane showed that certain ionic components of amosite have the capacity to cause a considerable

perturbation of the integrity of the macrophage membrane; this technique may be used further to estimate the effectiveness of the various ionic components of asbestos to modify the ionic transport system of the membrane, together with a comparison of any reductions in the efficient functioning of these cells.

In this study, an examination of the susceptibility of the asbestos-activated macrophage to a second challenge with asbestos dust in vitro has shown that these cells possess an enhanced susceptibility to the cytotoxic action of chrysotile. The reason for this enhanced susceptibility has not been shown, although it must be considered that the release of toxic oxygen species by activated macrophages (Bryant et al, 1982) may contribute to this situation by damaging the cell membrane. It is evident therefore that an examination of the role of cellular activation in determining the cytotoxic susceptibility of macrophages is of importance, in particular with regard to the identification of any alterations in the structure of the cell membrane which may render it more susceptible to the action of a cytotoxic dust. This type of study could be carried out in conjunction with an examination of the involvement of toxic oxygen species in contributing to the enhanced cytotoxic effect. An examination of the response of the activated alveolar macrophage in a similar situation may provide further information regarding the contribution of the macrophage secretory products in inducing inflammation and fibrosis in asbestos-induced disease.

In conclusion, this study has shown that not only is the fibre length content of a fibrous sample important in determining its cytotoxicity towards macrophages, but the activational status of each cell within the macrophage population may pre-determine the overall susceptibility of the population. The relationship between asbestos fibres and the macrophage is complex, and future research should assist in further clarifying the mechanism of asbestos-induced cytotoxicity and its relationship with asbestos-induced disease.

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Cytotoxic Effect of Asbestos on Macrophages in Different Activation States

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The *in vitro* effects due to phagocytosis of asbestos by mouse peritoneal macrophages in various stages of activation have been compared. The amphiboles proved relatively inert; chrysotile, however, expressed a greater degree of cytotoxicity toward those populations of macrophages induced *in vivo* with asbestos, than toward any of the other populations of cells. These results are compared with data concerning the enzyme release from the different populations of macrophages following phagocytosis of asbestos. The results indicate that those macrophages that have been exposed to a prior stimulation of either amphibole or serpentine asbestos *in vivo* are particularly sensitive to exposure to a second dose of a toxic fiber.

Introduction

The realization that the alveolar macrophage is the first phagocytic cell in the lung to ingest inhaled asbestos fibers prompted considerable research into the direct effects of fibers on such cells *in vitro*. Early studies (*1*, *2*) have shown chrysotile to be more toxic than crocidolite or amosite, a finding confirmed by many research groups. Macrophage-activating agents, such as zymosan, are known to initiate selective release of lysosomal enzymes (*3*).

A study by Davies et al. (*4*) demonstrated that mouse peritoneal macrophages, upon phagocytosis of chrysotile *in vitro*, showed a selective release of lysosomal enzymes in the absence of cell death. However, Jaurand et al. (*5*) demonstrated an additional release of lactate dehydrogenase, thus suggesting some loss of viability, for alveolar macrophages exposed to chrysolite *in vitro*. Studies by Hamilton and colleagues (*6*, *7*) showed that macrophages exposed to chrysotile *in vitro* and *in vivo* release greater quantities of the neutral protease plasminogen activator. In addition, Miller (*8*) and Donaldson et al. (*9*) have shown that asbestos-induced macrophages obtained from either lung or peritoneal cavity possess an altered surface morphology and increased number of membrane receptors consistent with cell activation.

McGee and Myrvik (*10*) demonstrated that activated macrophages are more readily injured upon phagocytosis of toxic agents than resident cells. Wright and Davis (*11*) showed that chrysotile-activated macrophages are more sensitive to the cytotoxic action of chrysotile *in vitro* than unstimulated cells; in addition, Morgan and Allison (*12*) also suggested that macrophages elicited by different biochemical means may show a different response to a further stimulus. The aim of this study was to investigate further the response of populations of macrophages in different stages of activation to the action of asbestos *in vitro*, and enzyme release from these cells was also compared.

Materials and Methods

Stimulation and Harvesting of Peritoneal Exudate Cells (PEC)

Male CBA mice, 12 weeks old, were either untreated or injected intraperitoneally with one of the following stimulating agents: 1 mL saline (Dulbecco's A); 1 mL 10% protease peptone (Difco); 1.4 mg heat-killed *Corynebacterium parvum* (Wellcome), a well-known macrophage activating agent (*13*); 1 mL of 0.1% latex beads (0.81 μ) (Difco); or 2.5 mg of UICC crocidolite, UICC amosite or UICC chrysotile suspended in 1 mL saline.

Three days following injection, the mice were killed by ether overdose. The PEC were harvested by peritoneal lavage and washed.

Spreading Assay to Assess Degree of Macrophage Activation

PECs 1×10^5 were cultured on 6×22 mm glass coverslips in Ham's F10 medium (F10) + 20% fetal calf serum (FCS) at 37°C. After precisely 1 hr, the coverslips were washed vigorously to remove non-adherent cells. The remaining adherent macrophages were stained by May Grunwald and Giemsa stains. The relative degree of activation of the population was expressed in terms of the percentage of cells completely spread. This means of assessment has been shown to correlate with other methods of activation measurement such as Fc receptor and 5′-nucleotidase (*9*).

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Culture and Treatment of PEC Populations

After harvesting, 1×10^6 PECs were cultured in 35 mm dishes in F10 + 20% FCS. After 1 hr, the cells were washed with saline to remove nonadherents. The resulting macrophage populations were cultured in F10 + 20% FCS either untreated or treated with 0.1% latex beads (0.81 μ), or 100 μg UICC crocidolite, UICC amosite or UICC chrysotile per plate.

Assessment of Phagocytic Ability of Macrophages

The phagocytic ability of the macrophage populations was assessed microscopically after 24 hr culture with latex beads. A cell was termed phagocytic if it contained more than three latex beads.

Assessment of Viability of Macrophages

Viability of the cells was assessed, using Trypan Blue exclusion, 24 hr following treatment with latex, crocidolite, amosite or chrysotile.

Enzyme Assays

Lactate dehydrogenase (LDH) (*15*) and *N*-acetyl-β-*D*-glucosaminidase (glucosaminidase) (*16*) levels were assessed in both cells and culture medium after 24-hr culture with crocidolite or chrysotile.

In Vitro Activation of Macrophages by Lymphokine

Lymphokine, a known macrophage activating agent, was produced according to the method of Lazdins et al. (*17*) by exposing mouse splenocytes to 10 μg/mL of Concanavalin A (Con A) *in vitro* for 24 hr. Saline-induced macrophages were exposed to either the resulting lymphokine or a Con A supplemented control medium for 24 hr. The activated Con A control and untreated macrophages were then exposed to UICC chrysotile for a further 24 hr and their viabilities assessed.

Statistical Analyses

The data from the spreading assay, macrophage viability estimates and enzymes assays were examined by statistical analyses of variance, the within-experimental replication being used to provide estimates of random variation.

Results

All of the populations of PECs were found to contain 99% viable cells upon isolation from the groups of treated mice. The relative degree of activation of the adherent macrophages, according to their ability to spread on glass, is shown on Figure 1. A high degree of activation was found in those populations induced by asbestos and *C. parvum*, according to this method of activation assessment; they did not differ significantly in their ability to spread on glass. All of the remaining populations showed a much lower ability to spread on glass, the protease peptone population showing an increase over the unstimulated population ($p < 0.05$).

After 24-hr culture *in vitro* with latex beads, the macrophage groups were all found to contain 95% phagocytic and 95% viable cells following ingestion of latex beads. The effect of 24-hr incubation with chrysotile was very different from that of the other two types of asbestos (Figs. 2 and 3). Crocidolite and amosite proved noncytotoxic, and there were no significant differences observed between the macrophage populations ($p < 0.9$ overall). Chrysotile, however, while exhibiting a low degree of cytotoxicity towards the unstimulated and saline-induced populations, showed a slightly increased level of cytotoxicity towards the *C. parvum*-induced cells. All three types of asbestos-induced populations proved particularly susceptible to the cytotoxic action of chrysotile; viabilities of around 30% were obtained and no significant differences were found among these three populations. The populations of macrophages stimulated *in vitro* by Con A or lymphokine did not show an increased degree of susceptibility to the action of chrysotile (Table 1).

Ingestion of crocidolite, compared to control, stimulated an increased release of glucosaminidase in all cell populations ($p < 0.01$). An even more considerable release of this enzyme in all populations of cells followed chrysotile ingestion ($p < 0.01$) (Figs. 4 and 5). The level of release of LDH was lower than that observed for the glucosaminidase, although the asbestos-induced populations released a greater quantity of LDH than the unstimulated, saline and *C. parvum*-induced populations ($p < 0.025$).

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Discussion

Peritoneal macrophages can be obtained in a variety of states of activation possessing a variety of altered properties (16). In general, activated macrophages are larger, have more granules, spread to a greater extent on glass and have a greater capacity to kill microorganisms and tumor cells than unstimulated, resident cells (19). The degree of activation of the macrophage can vary considerably, depending on the nature of the stimulating agent; and this is illustrated in Figure 1 by using a single parameter for activation assessment. Studies (6, 9) have shown that intraperitoneal injection of asbestos can produce a population of viable macrophages with characteristics consistent with cellular activation. In the present study, crocidolite, amosite and chrysotile have all induced intraperitoneal populations of cells both viable and apparently activated to a degree similar to *C. parvum*-induced macrophages.

All the populations of cells showed a similarly high rate of phagocytosis, regardless of the activation state, and no cell death was observed because of ingestion of nontoxic latex beads alone. The amphiboles displayed a similar level of low cytotoxicity toward all types of macrophages (Figs. 2 and 3). These cells, however, showed a diverse response to chrysotile. The nonstimulated and saline-induced macrophages appeared resistant to the cytotoxic action of the dust, whereas the more activated populations showed an increased susceptibility, the asbestos-induced cells proving the most sensitive. These results agree with those of McGee and Myrvik (10), in that activated macrophages tend to lose viability more rapidly than nonstimulated cells upon phagocytosis of a toxic agent. The cells activated by lymphokine *in vitro* (Fig. 4) did not display an increased sensitivity to the action of chrysotile, thus suggesting that macrophages activated *in vivo* probably possess differing properties to those activated *in vitro*. It is of interest to note that, while the amphiboles—crocidolite and amosite—appeared relatively inert *in vitro*, both types of fiber have the capacity *in vivo* to induce macrophages that show a high sensitivity to the action of a cytotoxic dust. This is not due simply to an *in vivo* stimulating activity of particulate alone, as latex-induced macrophages did not display a high sensitivity to chrysotile.

The data regarding enzyme release from the macrophages (Figs. 4 and 5) agree with the finding of Hamilton (6), in that asbestos-induced macrophages secreted a similar quantity of lysosomal enzyme into the culture medium to that seen for the nonstimulated cells. Phagocytosis of crocidolite induced a slight release of glucosaminidase by all populations; however, chrysotile stimulated a large release of enzyme similar to that seen in other reports (20). This large release of lysosomal enzyme was not accompanied by a corresponding release of cytoplasmic LDH for unstimulated, saline and *C. parvum*-induced macrophages. However, an increased release of LDH was observed for the asbestos activated populations, corresponding to the increased loss of viability illustrated in Figure 3.

In conclusion, this report illustrates that asbestos-induced macrophages, upon phagocytosis of a second dose of dust, do not respond in a manner similar to that observed for other types of macrophage populations. This must be taken into consideration when investigating the effect of inhaled particles on macrophages in the lung. Such cells may already have received prior stimulation by other toxic agents or pathogens and also persistent exposure to different dusts, rather than the single dose often used in the *in vitro* situation.

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Table 1. Percentage viability of *in vitro* activated macrophages following 24-hr treatment with chrysotile.

In vitro treatment of saline induced macrophages	% viability following ingestion of chrysotile*
Untreated control	67.2 ± 7.0
Con A supplemented medium	69.5 ± 8.7
Lymphokine	69.8 ± 9.2

* % viability = (number of viable cells on chrysotile treated plate/number of viable cells on control plate) × 100. Results are means ± of three experiments ± SD.

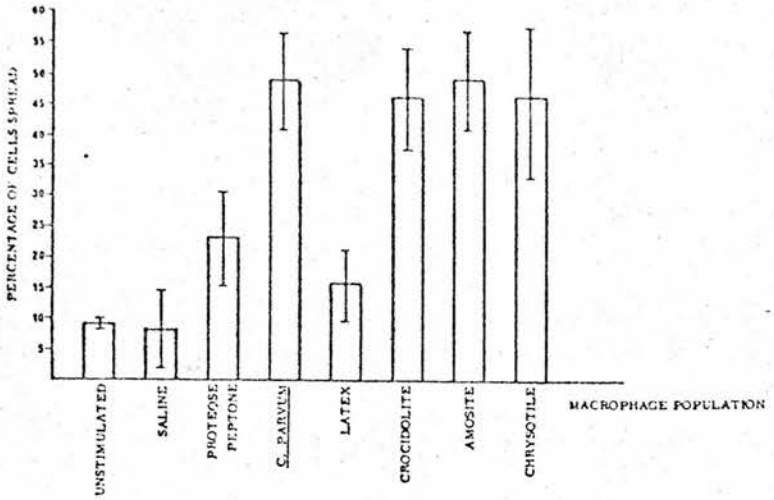
FIGURE 1. Relative degree of activation of macrophage populations according to spreading assay. Calculated as % cells spread = (number of cells spread/total number of cells counted) × 100. Results are means of at least three experiments ± SD.

FIGURE 2. Percentage viability of macrophage populations after 24 hr exposure to asbestos *in vitro*. Treatment *in vitro*: ○, crocidolite; ▽, amosite; □, chrysotile. Macrophages unstimulated or stimulated with saline, protease peptone and *C. parvum*. Viability calculated as % viability = (number of viable cells on treated plate/number of viable cells on control plate) × 100. Results are means of at least three experiments ± SD.

FIGURE 3. Percentage viability of macrophage populations after 24 hr exposure to asbestos *in vitro*. Treatment *in vitro*: ○, crocidolite; ▽, amosite; □, chrysotile. Macrophages stimulated with latex and asbestos. Results are means of at least three experiments ± SD.

FIGURE 4. Enzyme release into medium after 24 hr exposure to asbestos. Enzyme: ○, LDH; □, glucosaminidase. Treated with C = control, Cr = crocidolite, Ch = chrysotile. Results are means of three experiments ± SE.

FIGURE 5. Enzyme release into medium after 24 hr exposure to asbestos. Enzyme: ○, LDH; □, glucosaminidase. Treated with C = control, Cr = crocidolite, Ch = chrysotile.



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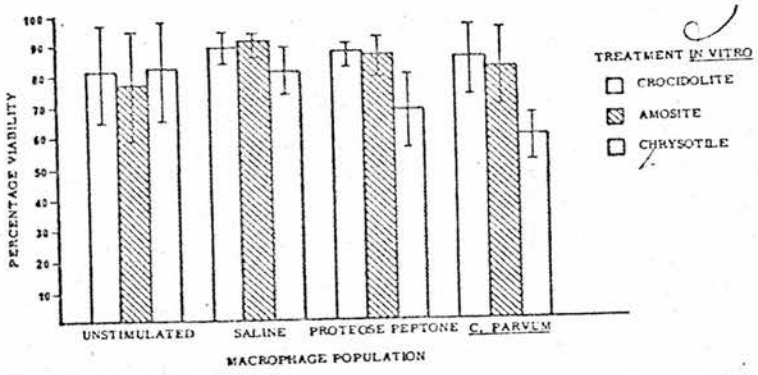

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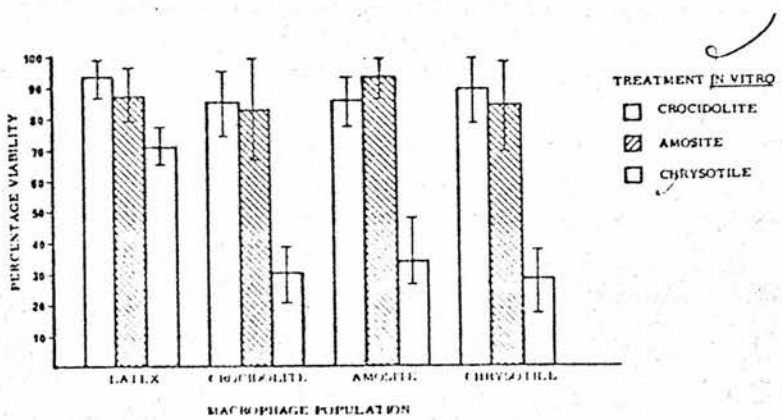

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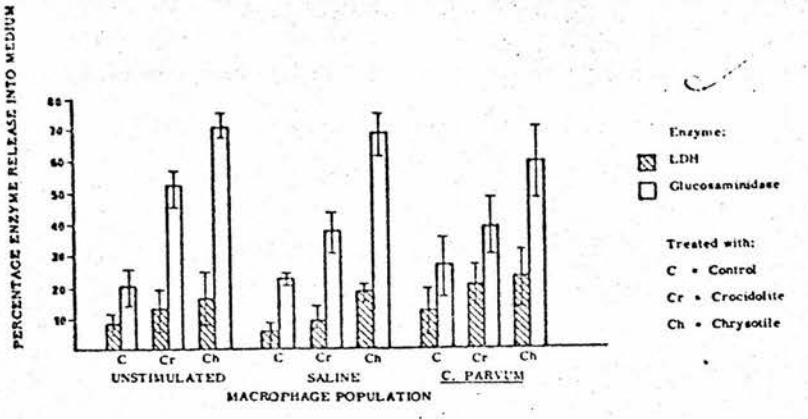
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C = Control
Cr = Crocidolite
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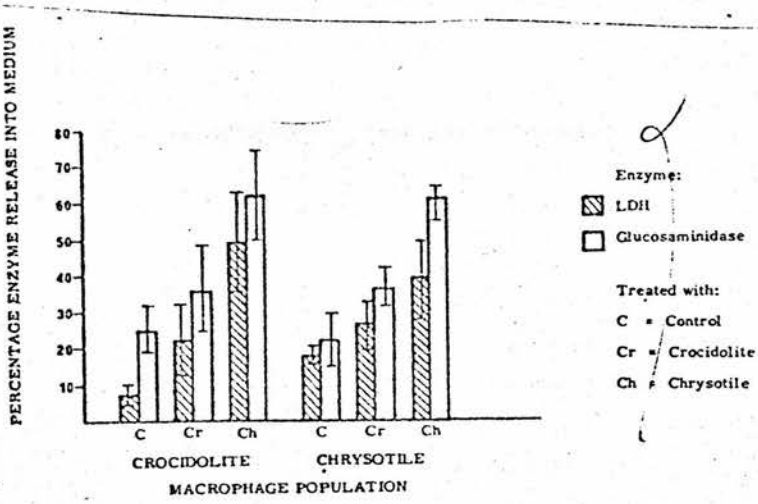
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THE CYTOTOXICITY OF UICC AND MODIFIED ASBESTOS FIBRES *IN VITRO*

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In spite of the known health hazards associated with the use of asbestos, its commercial value has ensured its continuing use. Different samples of asbestos are, however, likely to vary in their ability to induce fibrosis and/or malignant disease, and any such sample requires testing in order to determine its pathological potential. The traditional methods, using experimental animals, are both expensive and time consuming, and so there has been considerable interest in screening potentially pathogenic dusts and fibres *in vitro* and attempting to relate the results obtained to the *in vivo* situation.

In this investigation we have examined the cytotoxic and haemolytic potential of a number of asbestos samples. Their fibre length distributions have been investigated, as evidence has been accumulating that fibre size may be important in defining health hazards due to fibrous dusts (Stanton et al., 1977).

MATERIALS AND METHODS

The asbestos samples used in this investigation are detailed in Table 1.

Cytotoxicity was estimated using a permanent line of macrophage-like cells, $P388D_1$ (Dawe & Potter, 1957): 5×10^5 viable cells were exposed to 10 and 50 µg/ml of the sample (which was sonicated for two minutes to aid dispersion), for 24 and 48 hours at 37°C. The cultures were then assayed for cell viability, measuring trypan blue exclusion, lactate dehydrogenase (Wróblewski & Ladue, 1955) and *n*-acetyl-β-D-glucosaminidase (glucosaminidase) (Woolen et al., 1961).

The haemolytic activity of each dust was estimated using sheep erythrocytes, by a modification of the technique of Harington et al. (1971).

Fibre length distributions for each sonicated dust sample were determined using the scanning electron microscope (Schneider, 1978).

RESULTS

The haemolytic abilities of the samples tested are shown in Table 1.

Results of the cytotoxicity estimations using P388D_1 cells are shown in Table 2. Because of the variability between experiments and the design of the study, all six biochemical measurements were standardized before analysis. The logarithms of measurements were taken and an average value calculated from the four repeated biochemical measurements available for each dust concentration at each time point. Dust biochemical effects were then defined as the difference between this average and that of the similar average for titanium dioxide at the same time point. The statistical method of principal components (Seal, 1966) was then used to find linear functions of the six biochemical effects. The first principal component accounted for 62% of the variance of the dust biochemical effects. The value of this linear function was calculated for each dust for each concentration for each time point. This function is referred to as the 'cytotoxicity index'; it increases with increasing cytotoxicity and shows a high correlation (0.92) with percentage viability. It may be of interest that no evidence was found for a specific release of lysosomal enzymes in the present series of experiments, although Davies et al. (1974) observed this effect when mouse macrophages were treated with UICC chrysotile.

The fibre length distributions of the fibres used are shown in Figures 1-3, except that for wet-dispersed chrysotile which could not be measured accurately because the fibres tended to form aggregates which could not be broken up even by ultrasonic treatment.

DISCUSSION

The cytotoxicity of a number of asbestos fibres from different sources has been investigated using two different systems. Results concerning the ability of these fibres to damage erythrocytes (Table 1) were broadly in agreement with previous reports (see Harington, 1976 for review) in that all the amphibole fibres and the ceramic fibre tested showed little or no haemolytic ability. Only the bulk sample of UICC amosite caused measurable haemolysis (2.7%). In contrast,

Table 1. Samples of asbestos dust used in this study together with their haemolytic activity

Fibre type	Description of dust sample	% Haemolysis ± standard deviation[a]
(a) Amosite	UICC; sample for cytotoxicity tests taken from bulk material	2.7 ± 0.7
(b) Amosite	UICC; sample for cytotoxicity tests collected from an elutriated dust cloud produced in an experimental chamber	< 1
(c) Amosite	Collected from the filter bags of an asbestos factory	< 1
(d) Ceramic fibre (aluminium silicate)	Commercial insulation product; prepared as (b)	< 1
(e) Crocidolite	UICC; prepared as (a)	< 1
(f) Chrysotile	UICC; prepared as (a)	72 ± 1.3
(g) Chrysotile	UICC; prepared as (b)	85 ± 5.4
(h) Chrysotile	Commercial sample; heated to 850°C; sample for cytotoxicity tests prepared as (b)	34 ± 2.0
(i) Chrysotile	Parent to (h); sample prepared as (b)	67 ± 0.7
(j) Chrysotile	Collected from the filter bags of an asbestos factory	44 ± 1.3
(k) Chrysotile	Commercial material produced by a wet dispersion process; sample for cytotoxicity tests prepared as (b)	48 ± 11.4

[a] All results based on two independent experiments

all of the chrysotile samples caused extensive haemolysis, with the heated chrysotile being the least haemolytic (34%) and the elutriated UICC chrysotile being the most haemolytic (85%). The results obtained with the macrophage-like cell line, P388D_1 (Table 2) were generally in agreement with the results on haemolysis. The amphibole asbestos and ceramic samples showed little haemolytic or cytotoxic activity.

Table 2. The effect of asbestos samples on P388D$_1$ cells. The results, which are ranked by cytotoxicity, are expressed in terms of the cytotoxicity index, which is described in the text. Negative values indicate that the degree of cytotoxicity is low. Each result is based on at least two independent experiments.

10 µg sample/ml 24 hrs incubation	Index	50 µg sample/ml 24 hrs incubation	Index	10 µg sample/ml 43 hrs incubation	Index	50 µg sample/ml 48 hrs incubation	Index
Factory amosite	- 1.80	Elutriated UICC amosite	- 1.36	Elutriated UICC amosite	- 1.69	Ceramic	- 0.60
Elutriated UICC amosite	- 1.66	Factory amosite	- 1.12	Heated chrysotile	- 1.58	Elutriated UICC amosite	- 0.54
Heated chrysotile	- 1.50	Ceramic	- 0.87	Ceramic	- 1.24	Factory amosite	- 0.19
UICC crocidolite bulk sample	- 1.37	UICC amosite bulk sample	- 0.77	Factory amosite	- 1.08	Heated chrysotile	+ 0.16
UICC amosite bulk sample	- 0.85	Heated chrysotile	- 0.62	UICC amosite bulk sample	- 0.67	UICC crocidolite bulk sample	+ 1.12
Factory chrysotile	- 0.67	UICC crocidolite bulk sample	- 0.58	Parent heated chrysotile	- 0.35	UICC amosite bulk sample	+ 1.21
Parent heated chrysotile	- 0.62	Factory chrysotile	+ 0.02	UICC crocidolite bulk sample	- 0.32	Elutriated UICC chrysotile	+ 2.23
Ceramic	- 0.62	Parent heated chrysotile	+ 0.63	Elutriated UICC chrysotile	+ 0.04	Wet-dispersed chrysotile	+ 2.52
UICC chrysotile bulk sample	- 0.12	Elutriated UICC chrysotile	+ 0.81	Factory chrysotile	+ 0.32	Factory chrysotile	+ 2.62
Elutriated UICC chrysotile	- 0.05	Wet-dispersed chrysotile	+ 1.02	UICC chrysotile bulk sample	+ 0.96	Parent heated chrysotile	+ 2.69
Wet-dispersed chrysotile	+ 0.23	UICC chrysotile bulk sample	+ 1.11	Wet-dispersed chrysotile	+ 2.38	UICC chrysotile bulk sample	+ 2.77

FIG. 1. LENGTH DISTRIBUTIONS OF FIBRES OF CHRYSOTILE DUST SAMPLES USED IN THE PRESENT STUDY, FROM SCANNING ELECTRON MICROSCOPE MEASUREMENTS

■ - factory chrysotile, 0.4 µm median diameter; □ - UICC chrysotile, 0.3 µm median diameter; O - elutriated UICC chrysotile, 0.2 µm median diameter

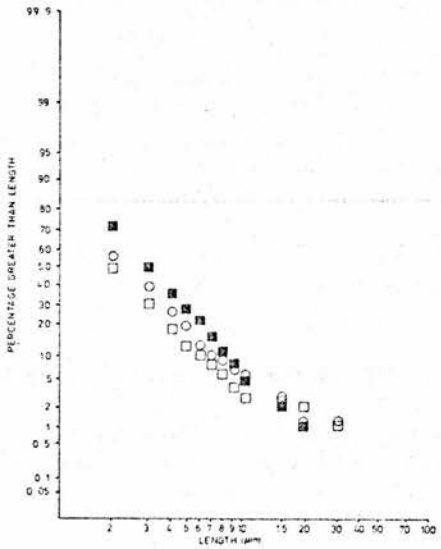


FIG. 2. LENGTH DISTRIBUTIONS OF FIBRES OF AMOSITE DUST SAMPLES USED IN THE PRESENT STUDY, FROM SCANNING ELECTRON MICROSCOPE MEASUREMENTS

■ - factory amosite; □ - UICC amosite; ○ - elutriated UICC amosite; all 0.4 μm median diameter

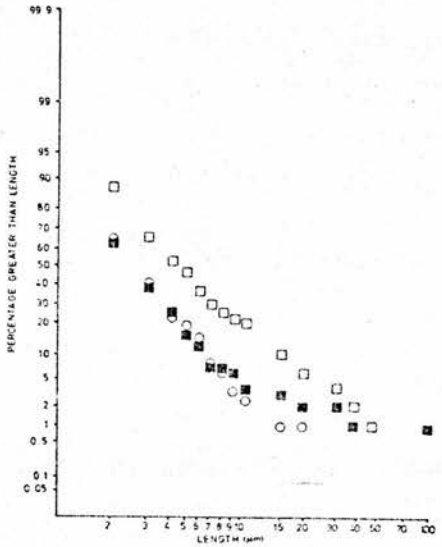
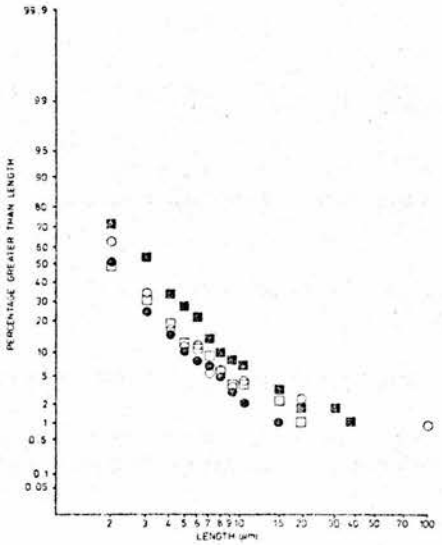


FIG. 3. LENGTH DISTRIBUTIONS OF FIBRES OF OTHER DUST SAMPLES USED IN THE PRESENT STUDY, FROM SCANNING ELECTRON MICROSCOPE MEASUREMENTS

■ - heated chrysotile, 0.5 μm median diameter; □ - parent of heated chrysotile, 0.3 μm median diameter; ● - UICC crocidolite, 0.3 μm median diameter; ○ - ceramic, 0.6 μm median diameter



The chrysotile samples, however, caused extensive cell damage in both systems, although heating a sample to 850°C markedly decreased its haemolytic and cytotoxic potential. There were, however, a number of discrepancies between the two sets of results. Although the UICC chrysotile sample prepared from bulk material was one of the most cytotoxic and haemolytic dusts examined, the UICC chrysotile sample collected from an experimental dust cloud was slightly less cytotoxic but more haemolytic than the original sample. The results for the most cytotoxic dust, the chrysotile prepared by wet dispersion, should be considered a conservative estimate, as this substance was not only difficult to disperse but tended to re-aggregate in the cultures. This phenomenon was most pronounced when the preparation was tested in the haemolytic system; the increased standard deviation partially reflects this problem.

The data in Table 2 show that the damage caused in $P388D_1$ cells by all the samples, with the exception of ceramic which was almost inert, increased with both time and dose. Even the amphibole samples caused some cytotoxicity after the cells had been exposed to 50 µg/ml of the sample for 48 hours. This protocol was found to be the most useful in distinguishing between samples of low cytotoxicity, but the lower doses and shorter times were useful in the identification of the most cytotoxic dusts. For example, wet-dispersed chrysotile was clearly the most cytotoxic dust when 10 µg/ml was the dose used.

This study has produced little information on the effect of length on the cytotoxicity of chrysotile fibres, since all the samples had a similar fibre length distribution. However, the UICC amosite sample produced from bulk material did have a higher proportion of long fibres and was slightly more haemolytic than other amosite samples as well as being the most cytotoxic after 48 hours of incubation with $P388D_1$ cells.

The results from tests of both haemolysis and *in vitro* cytotoxicity were broadly in agreement with those of other workers (Harington, 1976). Most previous workers have used macrophages for cytotoxicity tests with dust particles. However, since the $P388D_1$ cells are a permanent line which can be maintained *in vitro* indefinitely, the production of sufficient cells for multiple cytotoxicity tests is simpler than repeated extraction of macrophages from animals, and in our experience the results are more reproducible.

In our laboratory, a parallel series of experiments has been undertaken whereby the same dust samples are injected intraperitoneally into rats, in order to determine whether or not cytotoxicity *in vitro* correlates with neoplastic potential. Injection of aluminium silicate fibres was undertaken later than that of the other dusts, and results are not yet available. In addition, the dose chosen (25 mg) was probably too high to separate the effects of closely related dusts, and all samples except one produced mesotheliomas in over 90% of animals. However,

the wet-dispersed chrysotile, which was the most cytotoxic sample, produced tumours several months earlier than any other dust; and the heated chrysotile, which showed low levels of cytotoxicity, produced tumours later than other dusts and in only 30% of animals. There is thus a suggestion that cytotoxicity and carcinogenicity may be related, but the tumour production studies need to be repeated using a wider range of doses to see if this relationship holds for all dusts.

SUMMARY

Samples of asbestos were tested for their potential to damage $P388D_1$ cells and erythrocytes. The results obtained using the two systems were generally in agreement, with the amphibole being less cytotoxic and haemolytic than the chrysotile samples. Fibre length distributions for the chrysotile samples were relatively similar, so that, for this material, cytotoxicity and fibre length could not be correlated. However, some relationship was seen with amosite samples. Although there was agreement in some cases between cytotoxicity *in vitro* and the numbers of mesotheliomas produced in rats during a separate study using the same dusts, this relationship was not obvious for many of the samples tested.

RESUME

Les auteurs ont testé des échantillons d'amiante afin de déterminer leur pouvoir d'altérer les cellules $P388D_1$ et les erythrocytes. Les résultats obtenus dans les deux systèmes concordaient généralement, l'amphibole s'avérant moins cytotoxique et hémolytique que le chrysotile. Dans les échantillons de chrysotile, les distributions des longueurs des fibres étaient relativement semblables, en sorte que, pour cette substance la cytotoxicité et la longueur des fibres n'ont pu être mises en corrélation. On a cependant observé une certaine relation pour les échantillons d'amosite. Bien qu'on ait constaté, dans certains cas, une concordance entre la cytoxicité *in vitro* et le nombre de mésothéliomes provoqués chez le rat lors d'une étude distincte utilisant les mêmes poussières, pour beaucoup des échantillons testés cette relation n'était pas évidente.

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CONTACT GUIDANCE OF LOCOMOTION IN HUMAN NEUTROPHIL LEUCOCYTES

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E.M.C.

The influence of the alignment of the substratum on the direction of locomotion of human neutrophils has been studied in two systems, (a) the grooves of serum-coated glass Neubauer counting chambers, and (b) within the matrix of aligned collagen gels. If human neutrophils were allowed to adhere to a counting-chamber grid in 20% plasma at 37°C and their locomotion was filmed, it could be seen that, when cells moving on plane glass met a groove, they were highly likely to join and migrate along the groove. Of 33 cells filmed in such an experiment, 23 settled on plane glass and 10 on grooves. Fifteen minutes later, only two cells were still on plane glass, the rest having joined grooves. Neutrophils were filmed moving within aligned 3-D collagen gels. Analysis of the tracks of these cells showed a significant bias towards locomotion in the axis of alignment compared to other directions. The cells were not constrained to the line of alignment of the fibres and they could move across that line, but they spent more time moving in directions close to the angle of alignment than in other directions. We conclude that the direction of neutrophil locomotion is guided by the three-dimensional shape of the substratum, and suggest that this may be an important determinant of their movement through structured tissues *in vivo* e. g. in inflamed sites.

THE TOXICITY OF ASBESTOS TO MACROPHAGES IN VARIOUS ACTIVATION STATES.

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Alveolar macrophages are the main phagocytic cells involved in defense of the lung. Macrophage injury is thought to be a major causative factor in the formation of granulomas in such diseases as tuberculosis (Adams,D.O.1976;Am.J.Pathol. 84,1:164-191), and fibrogenesis associated with silicosis and asbestosis (Allison, A.C.1971; Arch.Intern.Med.,128, 131-139.) It has been suggested that activated macrophages are more readily injured upon phagocytosis of certain particulates than resident cells (McGee,M.P. and Myrvik,D.N. 1979;Infect.Immun.26,3:910-915), and lytic enzymes capable of extensive tissue damage may be released upon macrophage injury. Macrophages can be obtained in various stages of stimulation or activation depending on the degree of exposure of the host to previous stimulating agents and pathogens. This study examines the cytotoxic effect of asbestos fibres *in vitro* on these different populations of cells.

Populations of mouse peritoneal marcophages were induced *in vivo* using the following agents: saline, protease peptone, *C.parvum* and asbestos. The populations were exposed to asbestos *in vitro* for 24 hours and their viabilities assessed using Trypan Blue exclusion. Viability counts indicated that the susceptibility of the macrophages to the cytotoxic action of chrysotile asbestos increased with the degree of activation of the cell; the asbestos-induced macrophages proving by far the most sensitive *in vitro*.

4. THE CYTOTOXICITIES OF ASBESTOS AND OTHER FIBROUS DUSTS

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Abstract

A variety of fibrous dusts were assayed for their ability to lyse erythrocytes and damage P388D$_1$ cells *in vitro*. The results from the 2 assays were in general agreement, the amphiboles being less cytotoxic and haemolytic than the chrysotile samples. There were, however, a number of discrepancies between the 2 systems. Fibre length distributions suggested that samples with shorter, finer fibres were markedly haemolytic, although these samples did not show a corresponding cytotoxicity.

Introduction

In this study the haemolytic and cytotoxic potential of a variety of fibrous dusts have been investigated. The fibre length distributions have also been assessed as recent reports suggest that fibre length can affect the pathogenic (Stanton *et al.*, 1977) and cytotoxic (Brown *et al.*, 1978) ability of a dust sample.

Materials and Methods

The fibrous samples studies are detailed in Table 1.

The haemolytic ability of each dust was estimated using a modification of Harington *et al.*'s (1971) technique.

The cytotoxic activity was estimated using the macrophage-like cell line, P388D$_1$ (Dawe and Potter, 1957). 5×10^5 viable cells were exposed to 10 and 50 μg/ml of dust (which was sonicated to aid dispersion). After 24 and 48 hours the cultures were assayed for cell viability using trypan blue exclusion, lactate dehydrogenase release (Wróblewski and Ladue, 1955), N–acetyl–β–D–glucosaminidase (Woollen *et al.*, 1961), lactic acid (Gutmann and Wahlefield, 1974)

and cellular protein content (Chaykin, 1966). The controls consisted of undusted cells and cells exposed to the non-toxic dust titanium dioxide.

Fibre length distributions for each sonicated sample were determined using the scanning electron microscope (Schneider, 1978).

Results

The haemolytic abilities of the samples are shown in Table 1. The

TABLE 1

Samples of Fibres Used in this Study and Their Haemolytic Activity

	FIBRE TYPE	DESCRIPTION	% HAEMOLYSIS ± STANDARD DEVIATION ALL RESULTS BASED ON TWO INDEPENDENT EXPERIMENTS
a)	UICC CROCIDOLITE	Taken from bulk UICC material	0
b)	UICC AMOSITE	Taken from bulk UICC material	2.7 ± 0.7
c)	ELUTRIATED UICC AMOSITE	Fibres collected from an elutriated dust cloud produced in an experimental chamber	0
d)	FACTORY AMOSITE	Collected from the filter bags of an asbestos factory	0
e)	UICC CHRYSOTILE	Taken from bulk UICC material	72 ± 1.3
f)	ELUTRIATED UICC CHRYSOTILE	Prepared as in c)	85 ± 1.7
g)	HEATED CHRYSOTILE	A commercial sample, heated to 850°C, then prepared as in c)	34 ± 2.0
h)	PARENT OF THE HEATED CHRYSOTILE	Prepared as in c)	67 ± 0.7
i)	FACTORY CHRYSOTILE	Collected from the filter bags of an asbestos factory	44 ± 1.3
j)	SFA CHRYSOTILE	Donated by MRC Pneumoconiosis Unit, Cardiff	34 ± 4.7
k)	WET DISPERSED CHRYSOTILE	Commercial material produced by a wet dispersion process. Sample then prepared as in c)	48 ± 11.4
l)	CERAMIC FIBRE	A commercial insulation product (aluminium silicate) prepared as in c)	0.7 ± 0.7
m)	SEPIOLITE	A commercial product	88 ± 1.7
n)	CALCIUM SILICATES:	Commercial insulation products	
	SAMPLE 1		92 ± 2.0
	SAMPLE 2		94 ± 1.3
	SAMPLE 3		94 ± 0.7

TABLE 2

The Effect of Fibrous Dusts on P388D$_1$ Cells. The Results, Ranked by Cytotoxicity, are Expressed in Terms of the Cytotoxicity Index (see Text). Negative Values Indicate that the Degree of Cytotoxicity is Low. Each Result is Based on at least 2 Independent Experiments

24HRS INCUBATION 10 μg/ml sample	INDEX	24HRS INCUBATION 50 μg/ml sample	INDEX	48HRS INCUBATION 10 μg/ml sample	INDEX	48HRS INCUBATION 50 μg/ml sample	INDEX
Factory amosite	−1.99	Factory amosite	−1.38	Elutriated UICC amosite	−2.12	Ceramic	−1.10
Elutriated UICC amosite	−1.59	Elutriated UICC amosite	−1.36	Heated chrysotile	−2.05	Elutriated UICC amosite	−0.96
Heated chrysotile	−1.45	Ceramic	−1.20	Ceramic	−1.68	Factory amosite	−0.51
Ceramic	−1.03	Heated Chrysotile	−0.70	Calcium silicate (3)	−1.55	Calcium silicate (3)	−0.41
Sepiolite	−0.83	UICC crocidolite	−0.48	Factory amosite	−1.54	Heated chrysotile	−0.26
UICC crocidolite	−0.74	UICC amosite	−0.24	Calcium silicate (2)	−1.13	Calcium silicate (2)	+0.49
Parent of heated chrysotile	−0.68	Calcium silicate (3)	+0.06	Calcium silicate (1)	−1.10	Sepiolite	+0.76
UICC amosite	−0.50	Factory chrysotile	+0.68	UICC amosite	−1.07	UICC crocidolite	+0.91
Factory chrysotile	−0.37	Sepiolite	+0.71	Parent of heated chrysotile	−0.79	UICC amosite	+0.94
SFA chrysotile	−0.23	SFA chrysotile	+0.98	UICC crocidolite	−0.69	Calcium silicate (1)	+1.05
Calcium silicate (1)	−0.05	Calcium silicate (1)	+1.00	Sepiolite	−0.54	SFA chrysotile	+1.16
Elutriated UICC chrysotile	+0.09	Parent of heated chrysotile	+1.06	SFA chrysotile	−0.53	Elutriated UICC chrysotile	+1.84
UICC chrysotile	+0.24	Calcium silicate (2)	+1.10	Elutriated UICC chrysotile	−0.43	Wet dispersed chrysotile	+2.11
Calcium silicate (2)	+0.52	Elutriated UICC chrysotile	+1.46	Factory chrysotile	−0.11	Factory chrysotile	+2.17
Calcium silicate (3)	+0.55	UICC chrysotile	+2.05	UICC chrysotile	+0.53	UICC chrysotile	+2.21
Wet dispersed chrysotile	+1.50	Wet dispersed chrysotile	+2.80	Wet dispersed chrysotile	+1.97	Parent of heated chrysotile	+2.41

cytotoxic activity of each dust towards P388D$_1$ cells expressed in terms of a cytotoxicity index is shown in Table 2. Given the variability between experiments, average biochemical measurements have been standardised by defining biochemical effects due to the dust as the difference between this average (on a logarithmic scale) and that of a similar average for the appropriate titanium dioxide control. The statistical method of principal components (see e.g. Seal, 1966) was used to find linear functions of the effects, separate analyses being carried out for 24 and 48 hour results. The first component, referred to as the cytotoxicity index, accounted for 56.2% of the observed variability of the effects for the 24 hour analysis, the proportion was 70.3% for the 48 hour series. The value of the index was calculated for each dust/concentration/time combination. The index increases with increasing cytotoxicity and showed a high correlation with cell viability at 24 hours ($r = -0.82$) and at 48 hours ($r = -0.95$).

Fibre length distributions for a selection of the samples are shown in Figures 1–3.

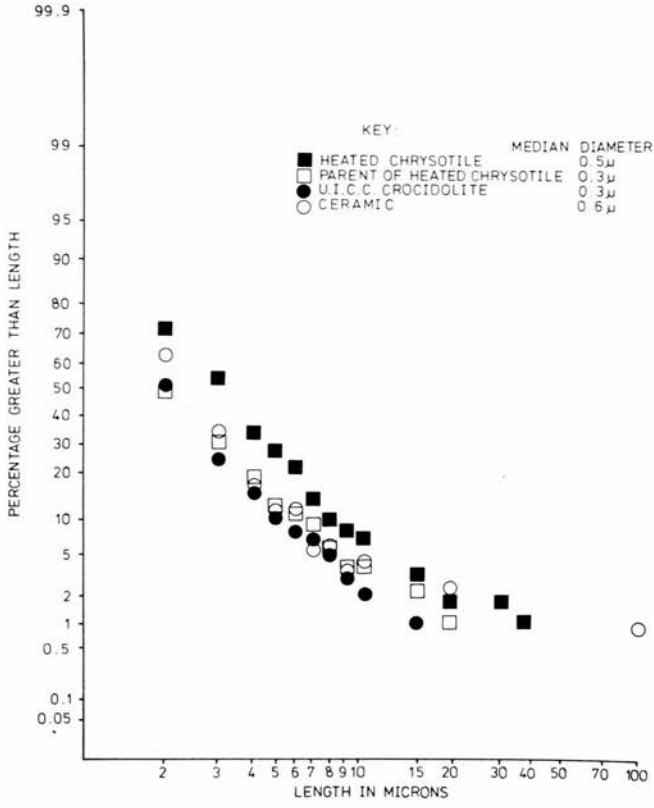

Fig. 1

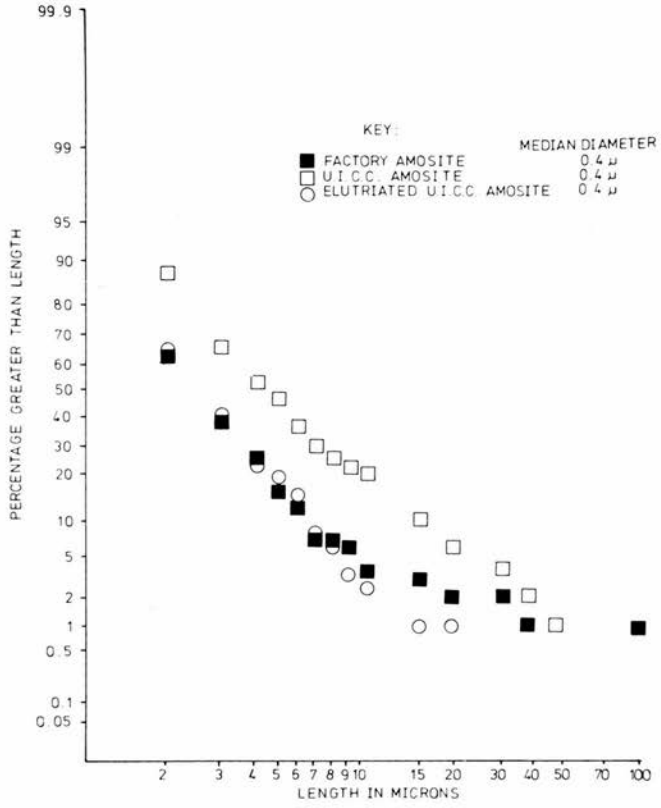

Fig. 2

Discussion

The results from this study were generally in agreement with previous reports; the amphiboles proving less haemolytic and cytotoxic than the chrysotile samples (Harington, 1976). The results for the asbestos samples showed that the cytotoxic and haemolytic ability of UICC amosite declined after elutriation and this process reduced the number of longer fibres (Fig. 2). However, elutriation of the UICC chrysotile sample resulted in a reduction in cytotoxicity but an increase in the haemolytic ability, although there was little apparent difference in fibre length distribution (Fig. 3). This may reflect the inability of the SEM to resolve small fibres and suggests that a TEM distribution may also be required when very fine fibres are present.

One of the most cytotoxic samples studied, wet dispersed chrysotile, did not show a correspondingly high haemolytic ability. This was probably due to the marked reaggregation of the fibres which

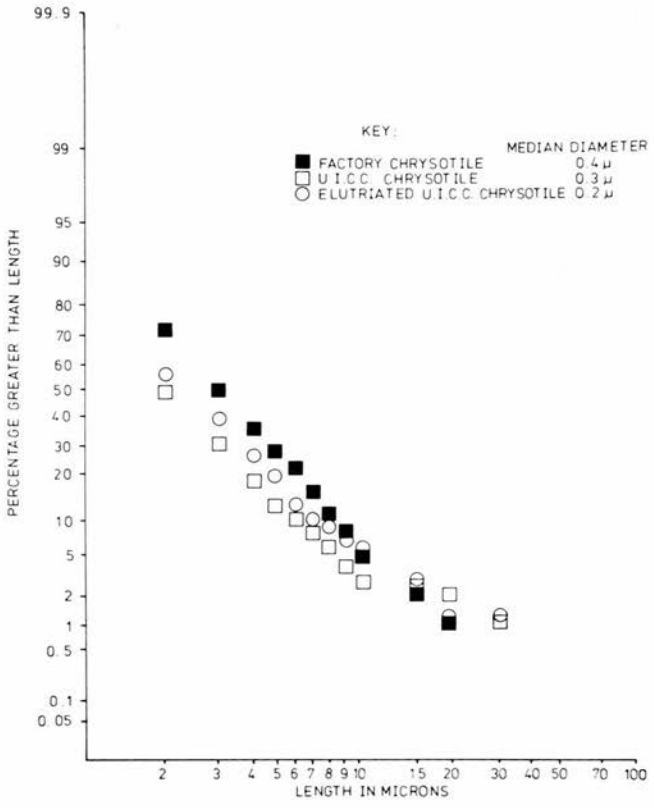

Fig. 3

Figs. 1, 2 and 3 Length distributions of fibres from the dust samples used in the present study. (Scanning electron microscope measurements of 200 fibres/sample).

was observed in the haemolysis tubes. This reaggregation would prevent adequate fibre/erythrocyte interaction and therefore haemolysis. The sample of chrysotile heated to 850°C showed a markedly reduced haemolytic and cytotoxic ability relative to its parent. However, the heating process had greatly reduced the number of fibres per unit mass of dust and many of those remaining were of relatively large diameter (Fig. 1).

The sample of ceramic dust which contained long but relatively thick fibres (Fig. 1) proved comparatively non-haemolytic and non-cytotoxic. The most marked discrepancies between the 2 assay systems occurred with sepiolite and the calcium silicates. They were the most haemolytic dusts tested but did not show a correspondingly high cytotoxicity. These samples, which were composed of very small

fibres and particles (sepiolite $< 4\ \mu m$ long; calcium silicates $< 1-2\ \mu m$ long) have a large number of particles/unit weight present and this might explain the marked haemolytic effect (Ottery and Gormley, 1978).

The results of this study suggest that the use of different time points and sample weights help to define the cytotoxicity of a dust, for example the 10 μg/ml sample at both time points clearly showed wet dispersed chrysotile to be very cytotoxic whereas the least cytotoxic dusts were most easily distinguished using 50 μg/ml of sample at 48 hours. The results also suggest that although general agreement between the cytotoxicity and haemolysis assays was observed, marked discrepancies occurred, suggesting that fibrous dusts should be subjected to more than one assay system when attempting to relate an *in vitro* effect to potential pathogenicity.

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We are grateful to Mrs. G.M. Brown and Mr. J. Whitelaw for their skilled technical assistance.

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SOME OBSERVATIONS ON THE IN VITRO CYTOTOXICITY OF CHRYSOTILE PREPARED BY THE WET DISPERSION PROCESS

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ABSTRACT

Samples of chrysotile taken during and after treatment by the wet dispersion process have been tested for their cytotoxic effect *in vitro* and the results compared with both a UICC chrysotile A sample and a dust prepared from a standard chrysotile textile yarn. The results obtained from three assay systems, utilising $P388D_1$, V79-4 and A549 cells, indicated that most of these samples are more cytotoxic than the standard chrysotile controls. Preliminary results from a parallel *in vivo* study suggest that these samples are also more active in producing mesotheliomas in rats.

INTRODUCTION

In industrialised countries, man is continuously introducing alternative processes for the production and exploitation of new mineral substances. In many cases, the hazards to health resulting from the use of minerals are well established but, when new minerals or processes are involved, their potentially pathogenic effect is unknown. As epidemiological studies often take many years to complete, during which time a considerable number of people are exposed to a possibly hazardous situation, much effort has been extended in investigating short term laboratory techniques that are capable of detecting pathogenic minerals. These techniques have involved both *in vivo* and *in vitro* approaches.

The effects of mineral dusts in animals, mainly rats, have been extensively studied, with the animals being exposed to a given dust by inhalation or intratracheal instillation and then investigated for disease over their life span. As macrophages are considered to be the cells most likely to first come into contact with mineral particles in the lung, most of the *in vitro* assays have been based on the cytotoxicity of a given mineral for these cells. Both animal macrophages (MARKS, 1957; MARKS and NAGELSCHMIDT, 1959; VIGLIANI *et al.*, 1961) and more recently permanent lines of macrophage like cells (WADE *et al.*, 1976; GORMLEY *et al.*, 1979; DANIEL and LE BOUFFANT, 1980) have been used to investigate the cytotoxicity of mineral dusts and this effect seems to give an indication of the fibrotic potential of a given mineral dust. In addition, it has been suggested that assays using phagocytic cells can also be used to give an indication of the malignant potential of such dusts (WADE *et al.* 1980; BECK and TILKES, 1980). New forms of cytotoxicity assay have also been developed by CHAMBERLAIN and BROWN (1978) using other types of permanent cell lines and it is suggested that these indicate the carcinogenic potential of such dusts rather than their fibrogenicity.

The wet dispersion process is now commonly used by industry to produce an asbestos fabric from chrysotile and an experimental sample of this material was

found to be particularly cytotoxic in an *in vitro* assay using $P388D_1$ cells, a mouse macrophage like cell line (WRIGHT *et al.*, 1980). This material was subsequently found to induce mesotheliomas in rats at an earlier time than UICC chrysotile A (BOLTON *et al.*; in press). It seemed possible that these phenomena might be related to wet dispersion processes in general and so the present study was undertaken using several different samples, some of which had been treated in order to observe the effects of extraneous chemicals used during the chrysotile treatment.

MATERIALS AND METHODS

Dust samples

The wet dispersion (WD) process is one used by a number of manufacturers in order to prepare a particularly durable chrysotile yarn that can be spun or woven into cloth. Although the technique varies slightly from manufacturer to manufacturer the basic process consists of the disaggregation, using a wetti agent, of bundles of chrysotile fibres into a slurry consisting of predominantl single fibrils. This slurry is then passed through fine nozzles and the addition of electrolytes at this point causes the fibrils to reaggregate with a very firm bonding between them. The resulting strands of yarn can then be woven or spun to produce asbestos textile products (HERON and HUGGETT, 1971). The samples used in the present study consisted of:-

1. The original experimental sample tested previously (WRIGHT et al., 1980) (Original W.D. Chrysotile (WDC)).
2. A finished textile yarn from a long running factory WD process which was milled in order to generate dust (Milled WDC).
3. A sample from the process in 2 above from which the detergent had not been removed (Unextracted WDC).
4. A sample of material from the process in 2 above from which all traces of the detergent had been removed by heat cleaning (Heat cleaned WDC).
5. A dust sample collected from the factory air at the plant producing sample 2. This sample was collected from a section of the factory where only WDC products were handled (Factory WDC).

The WDC samples were compared to both a UICC chrysotile A sample and to dust prepared from a standard chrysotile textile yarn made from exactly the sam type of chrysotile used in the W.D. process. All of the samples tested were elutriated before use in either in vitro or in vivo studies. A number of thes samples were extremely difficult to suspend in tissue culture medium due to their tendency to form tangled masses of fibres during the suspension process.

The samples used *in vitro* were therefore ultrasonicated and, in some cases, forced through a hyperdermic syringe needle in an attempt to disperse them.

Cytotoxicity Assays

(a) P388D$_1$ assay

The techniques used have been fully described previously (GORMLEY *et al.*, 1979; WRIGHT *et al.*, 1980). In brief 5 x 10^5 viable cells of the macrophage like cell line P388D$_1$ were exposed to 10 and 50 μg/ml of the dust in 5 ml of culture medium. After 24 and 48 hrs the cultures were assayed for cell viability using the trypan blue exclusion technique, lactate dehydrogenase release (WRÓBLEWSKI and LADUE, 1955), N-acetyl-β-D-glucosaminidase (WOOLEN *et al.*, 1961), lactic acid (GUTMANN and WAHLEFELD, 1974) and cellular protein content (CHAYKIN, 1966). The controls consisted of undusted cells and cells exposed to 80 μg titanium dioxide/ml ("non-toxic control") or 20 μg quartz DQ$_{12}$/m (ROBOCK, 1973) ("toxic control").

(b) V79-4 cell assay

This assay was carried out using the techniques described by CHAMBERLAIN and BROWN (1978).

(c) Giant cell formation in A549 cultures

This assay was also carried out using the techniques described by CHAMBERLAI and BROWN (1978). The measurements of cell size were made using a Graphic Instruments GDS1 image analyser. However, the W.D. chrysotile samples were very cytotoxic when added to these cells and so a higher cell inoculum (2.0 x 10^5 cells/25 cm^2 tissue culture flask) was used with lower dust concentrations (25 and 50 μg/ml).

In both the V79-4 and A549 assays a sample of UICC crocidolite was added as an additional control so that the results could be compared with those reported by CHAMBERLAIN and BROWN (1978).

Animal Experiments

The techniques used in this part of the study have been fully described by BOLTON et al. (in press).

The 5 WDC samples and the two chrysotile reference samples were each injected into groups of 32 8-10 week old male AF/HAN random bred SPF Wistar Laboratory Rats. The elutriated dust samples were dry heat sterilised at 60°C for 30 minutes, mixed with sterile Dulbecco's Phosphate Buffered Saline and 2 ml of PBS containing 25 mg of the dust were then injected intraperitoneally. The animals were observed daily and killed when distressed or moribund.

RESULTS AND DISCUSSION

The mean results from a series of experiments using the $P388D_1$ assay are shown in Table 1. The results for the biochemical estimations were in good agreement with those for the cellular viability as reported previously (WRIGHT et al., 1980; GORMLEY et al., 1980) and so only the viability results are reported. These are presented as a percentage of those found for the titaniu dioxide controls. It can be seen from Table 1 that, although there were slight differences (probably due to experimental variation) in the ranking of the dusts according to their cytotoxicity, 4 out of the 5 samples of WDC were more cytotoxic than the UICC chrysotile control. The only exception to this was the sample of unextracted WDC. It was noteworthy that three samples of the finished product - Heat cleaned WDC, milled WDC and the environmentally collected factory sample were always the most cytotoxic regardless of time or dose and these three samples reduced the viability to approximately half of that seen with the UICC sample.

The concentration of dust causing a 50% reduction in the cloning efficien of the V79-4 cells (CHAMBERLAIN and BROWN, 1978) was calculated for each dust sample and the results are shown in Table 2. The results are broadly in agreement with those obtained using the $P388D_1$ assay in that the unextracted WDC sample was the least cytotoxic whereas the Factory and Milled WDC were the most cytotoxic. The result for UICC Crocidolite was in good agreement with that originally described by CHAMBERLAIN and BROWN (1978) who reported an LD_{50} of 9 $\mu g/ml$ for this substance. However, quartz DQ_{12} was always found to be active in this system with a mean LD_{50} of 28.8 $\mu g/ml$ in contrast to the result reported for Min-U-Sil and South African Silica reported by CHAMBERLAIN and BROWN (1978).

The results obtained when the samples of WDC were tested for their abilit to cause giant cell formation in A549 cells are shown in Tables 3 and 4.

These results are broadly in agreement with those reported above with the unextracted sample having the least effect whilst the other four samples caused more giant cell formation than UICC chrysotile A or milled chrysotile. The quartz sample was not active in this assay system and so was in agreement with the results reported by CHAMBERLAIN and BROWN (1978). In contrast to their results, however, it should be noted that UICC crocidolite only had a minimal effect in our experiments. This result was to be expected in view of the increased cell number and decreased dust dose which was used in this study to overcome the marked toxicity of the WDC samples.

When the results of all three assay systems were considered, the unextracted WDC sample was always found to be the least active of the WDC samples. The remaining 4 samples were more active than either UICC chrysotile A or milled chrysotile in the $P388D_1$ and A549 assay and two samples - Factory and Milled WDC - were more cytotoxic than the standard chrysotile samples in all three assay systems.

Only preliminary data from the animal experimentation is currently available for comparison with the cytotoxicity results. The mean induction period for the first 15 tumours after intraperitoneal injections of 25 mg of the samples is shown in Table 5. This data suggests that three of the WDC samples induced tumours at a faster rate than either milled chrysotile or UICC chrysotile A and two of these, milled and factory WDC, caused a marked response in all three cytotoxicity assays. The third sample, original WDC, was more active than the chrysotiles in two out of the three assay systems.

The *in vivo* results for the remaining two WDC samples were not in agreement with the *in vitro* data, as heat cleaned WDC which was active *in vitro* had the longest tumour induction time and the unextracted sample, which was the least active of the WDC samples *in vitro* produced mesotheliomas in rats after the same approximate period as the standard chrysotile samples. Obviously a full

comparison of the *in vivo* and *in vitro* data can not be made until the *in vivo* experiments have been completed. There are, however, possible reasons for the discrepancies. The unextracted WDC was difficult to suspend for the *in vitro* work and separation of the fibres may have been incomplete whereas adequate separation for tumour induction may well have occurred during many months of residence in the animal tissues. Alternatively, the detergent materials present in this sample could have a protective effect in the short term *in vitro* tests but be removed during the longer term *in vivo* experiments. The discrepancy between the *in vitro* and *in vivo* data for heat cleaned WDC may be related to the heat treatment as there is evidence to suggest that this was carried out at a temperature high enough to break down some of the chrysotile to forsterite. Although chrysotile samples treated in this way exhibit a lower cytotoxicity (WRIGHT *et al.*, 1980) and a reduced carcinogenic potential (BOLTON *et al.*; in press), the two processes may not be directly related.

Wet dispersed chrysotile products are very hard wearing and, when handled, produce very little dust. They have, therefore, been considered to pose a reduced health hazard when compared to standard chrysotile materials. Evidence from the present studies would suggest, however, that any dust that is produced from wet dispersed chrysotile may be more dangerous.

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TABLE 1 Order of cytotoxicity of WDC samples based on viability estimations.

Sample at 10 $\mu g/ml$	Viability *	Sample at 50 $\mu g/ml$	Viability *
24 Hours			
Unextracted WDC	96 ± 14	Unextracted WDC	87 ± 9
UICC Chrysotile A	84 ± 6	UICC Chrysotile A	47 ± 8
Milled Chrysotile	79 ± 10	Original WDC	34 ± 3
Original WDC	68 ± 11	Milled Chrysotile	34 ± 14
Heat cleaned WDC	62 ± 10	Milled WDC	25 ± 4
Factory WDC	50 ± 8	Heat cleaned WDC	22 ± 7
Milled WDC	44 ± 6	Factory WDC	20 ± 8
48 Hours			
Unextracted WDC	75 ± 4	Unextracted WDC	59 ± 7
UICC Chrysotile A	52 ± 6	UICC Chrysotile A	21 ± 5
Milled Chrysotile	43 ± 12	Milled Chrysotile	18 ± 7
Original WDC	30 ± 11	Original WDC	17 ± 5
Factory WDC	25 ± 3	Factory WDC	10 ± 3
Heat cleaned WDC	25 ± 5	Milled WDC	8 ± 2
Milled WDC	22 ± 4	Heat cleaned WDC	6 ± 1

* Viability = $\frac{\text{no. live cells for test dust}}{\text{no. live cells for } TiO_2 \text{ control}}$ x 100% ± SD.

The dusts are ranked in order of increasing cytotoxicity.

TABLE 2 Cytotoxicity of WDC samples using the V79-4 Assay.

Sample	LD_{50} (μg of dust) *
Unextracted WDC	87.0 ± 19.1
Titanium Dioxide	84.8 ± 14.8
Original WDC	46.2 ± 39.2
Quartz DQ_{12}	28.8 ± 12.4
Heat cleaned WDC	20.4 ± 15.8
UICC Chrysotile A	13.5 ± 5.6
UICC Crocidolite	11.6 ± 2.3
Milled Chrysotile	9.7 ± 4.6
Factory WDC	8.4 ± 5.9
Milled WDC	5.0 ± 3.4

* The concentration of dust causing a 50% reduction in the cloning efficiency of V79-4 cells.

TABLE 3 Giant cell formation in A549 cells.

Sample at 25 μg/ml	Size μm ± SD	% of cells in size range			% of cells > 25 μm
		0–25 μm	25–40 μm	> 40 μm	
Titanium Dioxide	17.78 ± 2.40	100	0	0	0
Unextracted WDC	18.13 ± 3.11	98	2	0	2
Quartz DQ_{12}	18.18 ± 3.58	98	1	1	2
Undusted control	18.32 ± 2.19	99	1	0	1
UICC Crocidolite	19.22 ± 3.66	95	5	0	5
UICC Chrysotile A	20.68 ± 4.69	83	17	0	17
Milled Chrysotile	21.38 ± 5.19	78	22	0	22
Heat cleaned WDC	22.71 ± 5.09	74	26	0	26
Factory WDC	24.18 ± 5.71	56	44	0	44
Milled WDC	24.51 ± 5.82	60	39	1	40
Original WDC	25.08 ± 6.85	56	41	3	44

TABLE 4 Giant cell formation in A549 cells.

Sample at 50 μg/ml	Size μm ± SD	% of cells in size range			% of cells > 25 μm
		0–25 μm	25–40 μm	> 40 μm	
Quartz DQ_{12}	17.54 ± 5.68	99	1	0	1
Titanium Dioxide	17.80 ± 1.94	99	1	0	1
Undusted control	18.32 ± 2.19	99	1	0	1
UICC Crocidolite	19.19 ± 3.23	93	7	0	7
Unextracted WDC	19.75 ± 4.41	92	7	1	8
Milled Chrysotile	22.46 ± 4.19	72	28	0	28
UICC Chrysotile A	23.21 ± 5.43	69	31	0	31
Factory WDC	23.49 ± 6.36	63	36	1	37
Heat cleaned WDC	24.39 ± 5.99	57	41	2	43
Original WDC	24.72 ± 6.22	62	35	3	38
Milled WDC	26.96 ± 7.12	44	50	6	56

TABLE 5 Preliminary data on mesothelioma production by WDC samples in rats.

Sample	Mean tumour induction time for 15 tumours in each group (days)
Heat cleaned WDC	367
Milled chrysotile	357
Unextracted WDC	355
UICC Chrysotile A	354
Milled WDC	300
Factory WDC	282
Original WDC	276